OBITUARY NOTICE.

JOHN WEBSTER.

JOHN WEBSTER, who died on January 20th from sudden heart failure following influenza, was a member of an old Quaker family, of Birmingham, where he was educated at Mason's College. He was a Fellow of the Chemical Society, of the Society of Public Analysts (of whose Council he was at one time a member), and of the Royal Society of Medicine. He was a member of the Medico-Legal Society, and of its council at the time of his death, and was also a member of the Biochemical Society.

Mr Webster was a most experienced toxicological analyst. About 1900 he became assistant analyst to Sir Thomas Stevenson, who was then senior scientific analyst to the Home Office, and remained in association with him until his death in 1908. Sir William Willcox succeeded Sir Thomas Stevenson, and Mr Webster became assistant analyst to him; and in 1919, when Sir William Willcox retired, Mr Webster was appointed senior scientific analyst to the Home Office.

Mr Webster was an analyst in whom absolute reliance could be placed as regards accuracy, which was essential in work connected with poisoning cases where the life of an accused person might depend on the result of an analysis. His ability was shown in many of the most famous poisoning cases in recent criminal history; he was engaged in the scientific investigations connected with the trials of Seddon, Greenwood, Armstrong, Bywaters and Mrs Thompson, and Vacquier, as well as in a number of smaller investigations.

During the past twenty years he had been on the staff of the Pathological Chemistry Department of St Mary's Hospital, Paddington, and had held the position of pathological chemist to the Hospital.

His great analytical skill and experience were of the utmost value in the clinical investigation of the pathological problems associated with cases in the wards of the Hospital, and they furnished a reliable guide in diagnosis and treatment of many of the difficult cases.

Mr Webster was a most painstaking, careful and neat investigator. He would be seen at his best when carrying out the toxicological investigation of viscera where great difficulties were encountered in consequence of putrefactive changes. The extraction of chemical poisons such as vegetable alkaloids in such cases requires the utmost skill and experience, and at work of this kind he showed a masterly ability and a brilliant technique which had been acquired by long experience and meticulous care.

He would always be absolutely sure of the accuracy of his results and if necessary confirm them by repeated analyses.

His work on the excretion of arsenic after the administration of either arsenic by the mouth or an arseno-benzene derivative intravenously has been of the utmost value not only in toxicological investigations but in guidance as to the correct time for the administration of repeated doses of arseno-benzene compounds.

In the recent Armstrong case the amount of arsenic found by Mr Webster in urine passed three days after the taking of the poison enabled him to say that the amount administered three days previously was a possible fatal dose. He published several papers on research work, especially with reference to arsenical poisoning and the toxicology of salvarsan.

During the War Mr Webster's time was fully occupied by his toxicological work for the Home Office and his Pathological Chemical work at St Mary's Hospital. He did not work on trinitrotoluene poisoning. The "Webster Test" of the urine in this condition was discovered by Mr T. A. Webster working in conjunction with Dr H. H. Dale and Dr Barger[1]. The "Webster Test" has been, in a recent obituary notice, incorrectly attributed to the late Mr J. Webster, F.I.C., F.C.S.

During the past twenty years, while at St Mary's Hospital, Mr Webster had closely identified himself with the work of the Hospital and Medical School. He was ever ready to help in the teaching of Forensic Medicine and Chemical Pathology, and whenever his great experience was required in any analytical investigation he was always most willing to help any student in difficulties.

He was a Vice-President of the Students' Musical Society at St Mary's Hospital and had conducted Concerts for them. He also had been for some years a member of the Sancta Maria Lodge and during that time had held the post of Honorary Organist.

His kindly disposition had endeared him to many of his friends and colleagues at St Mary's Hospital, and he will be much missed by them. His death will be a great loss to toxicological science.

He leaves a widow and one son.

[1] Medical Research Council Special Report Series, No. 58, *TNT poisoning and the fate of TNT in the animal body.*

W. H. Willcox.

XXXVIII. THE USE OF THE HYDROQUINHYDRONE ELECTRODE FOR p_H DETERMINATION IN THE FLUIDS OF THE ORGANISM.

By FELIX GROSSMAN.

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(*Received November 26th, 1926.*)

In the beginning of the year 1925 I began the investigation of the applicability of Biilmann's [1921] quinhydrone electrode to determine the p_H of serum, cerebrospinal fluid and blood. There has since appeared a number of papers showing that there is a divergence of opinion as to the suitability of this electrode for these purposes. The different experiences have recently been reviewed by Mislowitzer [1926] who adheres to his opinion, expressed earlier [1925], that the quinhydrone electrode can be employed to determine the p_H of blood.

My first measurements with a quinhydrone electrode gave unsatisfactory results. The potential was quite unstable. By diluting the serum several times with physiological saline solution I obtained somewhat better results. However, having made several measurements which did not give consistent results, I gave up the quinhydrone electrode and tried the hydroquinhydrone electrode, also invented by Biilmann [1921]. The difference between the quinhydrone and the hydroquinhydrone electrodes is that, instead of quinhydrone, an excess of a quinol (hydroquinone) and quinhydrone mixture is used. Biilmann and Lund [1921] recommend 0·1 g. of quinhydrone and 1 g. of quinol for 15 cc. of fluid. Part of this mixture is dissolved, the rest remains as a precipitate. The platinum wire of the electrode must be almost completely immersed in the precipitate.

The reaction in such an electrode consists in the transformation of stable quinhydrone into stable quinol:

$$C_6H_4(OH)_2.C_6H_4O_2 + H_2 \rightleftarrows 2C_6H_4(OH)_2.$$

The electromotive force of the chain: Pt/quinol, quinhydrone, solution A/KCl/solution A, H_2/Pt, according to Biilmann and Lund [1921] = 0·6179 volts at $t = 18°$, instead of 0·7042 in the case of the quinhydrone electrode.

With serum the potential became rapidly stable and the values obtained for p_H were consistent. They differed, however, from those obtained with the hydrogen electrode (see Table I).

Table I.

Pig-serum.

$t = 22°$.

	Hydrogen electrode				Hydroquinhydrone electrode					
No.	Volts	Average	p_H	Average	Volts	Average	p_H	Average	Δp_H	p'_H
1	0·708*		7·93		−0·068†		7·67			
2	0·707	0·7075	7·91	7·92	−0·070	−0·687	7·70	7·68	0·19	7·87
3					−0·068		7·68			

Ox-serum.

$t = 18°$.

1	0·691		7·76		−0·066		7·63			
2	0·692	0·693	7·78	7·79	−0·067	−0·0667	7·65	7·64	0·18	7·82
3	0·695		7·83		−0·067		7·65			

* These values are for chain: Pt/H_2, serum/KCl/satur. KCl, Hg_2Cl_2/Hg.
† These values are for chain: Pt/quinol, quinhydrone, serum/KCl/satur. KCl, Hg_2Cl_2/Hg.

After satisfying myself that my determinations of the p_H of pig-serum with the hydrogen electrode were correct, I repeated the measurements on ox-serum with both electrodes. I again obtained with the hydroquinhydrone electrode a similar difference from those obtained with the hydrogen electrode. Having previously tested this electrode upon standard solutions with $p_H < 7$ and found it to be very exact, I proceeded to ascertain its behaviour with slightly alkaline but well buffered solutions.

For this purpose I prepared a series of Sørensen's phosphate solutions and determined their p_H values with a hydrogen, a quinhydrone and a hydroquinhydrone electrode respectively (see Table II).

I obtained, as seen from Table II, differences between p_H determined with the hydroquinhydrone electrode and the quinhydrone electrode, increasing as the fluid became more alkaline.

Upon the ground of these determinations I arrived at an empirical formula for the correction to be applied when using the hydroquinhydrone electrode on a fluid with $p_H > 7$, namely, $\Delta p_H = 0{\cdot}24 \times 2^{p_H - 7} - 0{\cdot}21$.

If we take into consideration $p_H = 7{\cdot}00$–$8{\cdot}00$, that is the widest limits in which p_H can balance in the serum, blood, or cerebrospinal fluid of men and animals, then the rectification Δp_H will vary from 0·03 to 0·21. For body fluids Δp_H will not on the average exceed 0·13.

These corrections are introduced in the tables, and it will be seen that the values with the hydrogen and the hydroquinhydrone electrodes then become almost identical.

I am now working on the theoretical confirmation of the rectification, Δp_H, introduced by me, as was done by La Mer and Parsons [1923] for the aberrations of the quinhydrone electrode in alkaline solutions.

A hydroquinhydrone electrode possesses many advantages over a hydrogen electrode for determining the p_H in serum, blood, etc., both as to simplicity and rapidity in making determinations. Further, there are many conditions under which the hydrogen electrode cannot be used at all.

Table II.

	Hydrogen electrode			Quinhydrone electrode			Hydroquinhydrone electrode			
No.	Volts	Average	p_H average	Volts	Average	p_H average	Volts	Average	p_H average	Δp_H average
	Mixture of equal volumes of $M/15$ Na_2HPO_4 and $M/15$ KH_2PO_4; according to Sørensen $p_H = 6{\cdot}80$.									
1	0·634			0·0680			−0·0180			
2	0·636	0·6347	6·79	0·0680	0·0680	6·81	−0·0185	−0·0182	6·81	0·01
3	0·634			0·0680			−0·0180			
	Mixture of 61 volumes of Na_2HPO_4 and 39 volumes of KH_2PO_4, with $p_H = 7{\cdot}00$.									
				$t = 18°$.						
1	0·646			0·0570			−0·0260			
2	0·648	0·647	7·00	0·0570	0·0570	7·00	−0·0270	−0·0267	6·97	0·03
3	0·647			0·0570			−0·0270			
	Mixture of 80 vol. of Na_2HPO_4 and 20 vol. of KH_2PO_4, with $p_H = 7{\cdot}36$.									
				$t = 19°$.						
1	0·669			0·0345			−0·0460			
2	0·671	0·670	7·38	0·0345	0·0347	7·38	−0·0465	−0·0465	7·28	0·10
3				0·0350			−0·0470			
	Mixture of 87 vol. of Na_2HPO_4 and 13 vol. of KH_2PO_4, with $p_H = 7{\cdot}60$.									
				$t = 19°$.						
1	0·683			0·0230			−0·0555			
2	0·680	0·681	7·56	0·0230	0·0232	7·56	−0·0560	−0·0557	7·43	0·13
3	0·680			0·0235			−0·0555			
	Mixture of 60 vol. of borate and 40 vol. of 0·1 N HCl, with $p_H = 8{\cdot}27$									
				$t = 18°$.						
1	0·719			−0·0150			−0·0850			
2	0·720	0·719	8·25	−0·0160	−0·0155	8·26	−0·0850	−0·0855	7·98	0·273
3	0·718			−0·0155			−0·0855			
	Mixture of 84 vol. of borate and 16 vol. of 0·1 N HCl, with $p_H = 8{\cdot}99$.									
				$t = 18°$.						
1	0·760			−0·0570			−0·1150			
2	0·762	0·7607	8·97	−0·0560	−0·0560	8·96	−0·1150	−0·1145	8·49	0·475
3	0·760			−0·0550			−0·1135			

I, therefore, made parallel determinations of the p_H of serum, blood and cerebrospinal fluid with the hydroquinhydrone and hydrogen electrodes. These are set forth in Table III.

We see that after introducing the correction for the hydroquinhydrone electrode, the results obtained with this electrode and those obtained with the hydrogen electrode, given in Table III, are almost identical. I made measurements in the following way: I poured from 1·5 to 3 cc. of physiological salt solution into a small electrode vessel and added a mixture of dry quinhydrone and quinol in quantities more or less such as I have mentioned above; I stirred the contents of the small vessel, poured in 1·5 cc. of blood—that is, diluted it from 1 : 1 to 1 : 2—stirred the contents of the vessel well and after about three minutes read the potential of this electrode. The more the blood is diluted, the better and the more rapidly the potential of the electrode becomes

stable. The relative amounts of quinhydrone and quinol need not necessarily be exactly those recommended by Biilmann. After one has made several measurements, one can judge sufficiently correctly by eye, but the greatest part of the short platinum wire must be immersed in the precipitate.

Table III.

	Hydrogen electrode			Hydroquinhydrone electrode				
No.	Volts	Temp.	p_H	Volts	Temp.	p_H	Δp_H	p'_H
				Human serum.				
1	0·6855	20	7·63	−0·0625	20	7·51	14·5	7·655
2	0·6820	18	7·61	−0·0580	18	7·50	14·5	7·645
3	0·6810	18	7·59	−0·0550	18	7·45	13	7·58
4	0·6820	20	7·56	−0·0560	20	7·40	12	7·52
5	0·6820	19	7·59	−0·0560	19	7·43	13	7·56
				Cerebrospinal fluid.				
1	0·6910	20	7·72	−0·0640	20	7·53	15	7·68
2	0·6900	20	7·70	−0·0625	19·5	7·51	15	7·66
3	0·6830	20	7·58	−0·0615	20	7·48	14	7·62
4	0·6840	18	7·64	−0·0550	18	7·45	13	7·58
5	0·6850	18·5	7·65	−0·0580	18	7·50	14·5	7·645
				Rabbit blood.				
1	0·678	17·5	7·55	−0·050	17·5	7·38	0·12	7·50
2	0·671	18	7·42	−0·048	18	7·33	0·11	7·44
3	0·6805	17·5	7·59	−0·054	17·5	7·45	0·13	7·57
4	0·673	17	7·48	−0·046	16·5	7·35	0·115	7·465
5	0·683	18·5	7·62	−0·056	18·5	7·46	0·13	7·60
				Human blood.				
1	0·669	18	7·39	−0·045	18	7·28	0·10	7·38
2	0·675	18·5	7·47	−0·047	18	7·31	0·11	7·42
3	0·668	18	7·37	−0·043	18	7·25	0·10	7·35
4	0·674	18	7·45	−0·050	18·5	7·34	0·115	7·455
5	0·678	18·5	7·53	−0·053	18	7·42	0·125	7·545

Making several measurements on the same blood, for instance that of the rabbit, diluted with 0·85 % NaCl solution, I obtained with the hydroquinhydrone electrode almost identical values, for instance, 7·52, 7·52, 7·54. The quinhydrone electrode in the case of serum as in that of blood gave improbable and drifting results.

I attempted too, to use with blood the quinoquinhydrone electrode also invented by Biilmann [1921] for which a mixture of quinone and quinhydrone is employed. The reaction consists in the transformation of stable quinone into stable quinhydrone:

$$2C_6H_4O_2 + H_2 \rightleftarrows C_6H_4(OH)_2.C_6H_4O_2.$$

This electrode is unsuitable for blood as it has a very drifting potential. It is also inferior to the hydroquinhydrone electrode for serum.

I have found that the quinhydrone, hydroquinhydrone and especially the quinoquinhydrone electrode, as pointed out by La Mer and Parsons, cannot be used for fluids with $p_H > 9$, because the quinol oxidises too rapidly.

I have tried the gold electrode recommended by Dixon and Quastel [1923], Corran and Lewis [1924] and Mislowitzer [1925] but have not found it satisfactory. Mislowitzer [1926] has also abandoned it in favour of platinum.

SUMMARY.

To determine the p_H in physiological fluids such as, for instance, serum, plasma, blood, cerebrospinal fluid, etc., the hydroquinhydrone electrode is more exact and simpler than the hydrogen electrode and more generally applicable and, if a correction varying with the alkalinity of the solution is applied, affords results which agree with those obtained with the hydrogen electrode.

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XXXIX. PHOSPHORYLATION OF PROTEINS.

By CLAUDE RIMINGTON.

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(*Received December 31st, 1926.*)

The peculiarity of the phosphoproteins in containing relatively large quantities of phosphorus, organically combined, and the unique importance of these substances in the nutrition of the young organism has led in the past to a good deal of speculation as to the mode of combination by which the phosphorus is held in the molecule, and also to attempts on the part of one or two workers to produce similar phosphorus-containing bodies by the action of various reagents upon proteins.

Liebermann [1888] and Pohl [1889] investigated the action of metaphosphoric acid upon proteins. Phosphorus oxychloride appears to have been used first by Bechhold [1901] who treated egg-albumin, dissolved in aqueous alkaline solution, with this reagent. There was, however, great development of heat during the addition of the phosphorus oxychloride and a consequent destruction of the protein. The resulting reaction-mixture was found to contain substances, some of which were precipitable by acid, which contained phosphorus, and exhibited properties characterising them as derived proteins resembling albumoses in complexity.

A modification of the method was attempted by Neuberg and Pollak [1910] and later by Neuberg and Oertel [1914], the phosphorus oxychloride being dissolved in chloroform. Lactalbumin, serum globulin and caseinogen were "phosphorised" in this way and the phosphorised products precipitated by acetic acid and washed until no free acid remained in the filtrate. They contained amounts of phosphorus varying from 1·28 % in lactalbumin to 1·77 % in serum globulin. These authors also made some experiments of a purely qualitative nature upon the behaviour of the compounds towards the digestive enzymes. Neuberg and Oertel [1914] also state that phosphorised globulin is coagulated by rennin, an observation which the present author has not been able to confirm, as in his experience the calcium salt of the phosphorised protein is already insoluble under the conditions in which the test would be carried out.

Undoubtedly the technique employed by Neuberg affords a method of bringing about organic combination of phosphorus with the protein, but no

conclusive evidence was brought forward that the products were free from admixture with inorganic phosphoric acid. The difficulty of removing small quantities of this substance from protein precipitates is very great, and it seems that a couple of reprecipitations, even accompanied by thorough washing, is insufficient to secure complete freedom from inorganic impurities.

In the present work, a method is used by which the progressive purification of the product is followed by determining the P/N ratio of the *filtrates* from each reprecipitation. The process of reprecipitation and washing is repeated until the P/N ratio of the filtrate remains constant and is then equal, it is found, to the P/N ratio of the precipitated substance itself. By this procedure absolute certainty is achieved that all the phosphorus remaining in the product is in definite chemical union, while at the same time none of the substance itself is used up in analyses during its purification—a point of undoubted advantage when small quantities of material are being dealt with.

The method of carrying out the phosphorylation is analogous to the Schotten-Baumann reaction for the benzoylation of organic substances in aqueous solution. By this means the high concentrations of alkali employed by Neuberg are avoided and the risk of destructive changes in the proteins thereby diminished, for it has already been shown by Rimington and Kay [1926] that under the action of 1 % sodium hydroxide at 37° caseinogen rapidly loses both phosphorus (as phosphoric acid) and ammonia.

The proteins used in this investigation are caseinogen, serum globulin, prepared from horse serum, carefully purified and subsequently denatured by alcohol, and "dephosphorised caseinogen," the phosphorus-free protein obtained by the action of 1 % sodium hydroxide for 24 hours upon caseinogen at 37°. The globulin was chosen for the following reasons. It was shown by Crowther and Raistrick [1916] and confirmed by Woodman [1921] that the globulins of serum, colostrum and of milk are identical, whilst the albumin of serum differs from that present in colostrum and milk. The proportion of lactoglobulin in colostrum is very high, 6–12 %, and falls immediately to the low figure of 0·005 % as soon as secretion of true milk commences.

In considering the problem of mammary secretion, the possibility has always to be kept in mind that one or other of the blood proteins might conceivably furnish the starting point for the synthesis of caseinogen. The ready permeability of the cells of the mammary gland to serum globulin, as evidenced by the identity of the protein in serum, colostrum and milk respectively, taken together with the difference in the amounts occurring in the two secretions makes this possibility worthy of further consideration. Moreover the distribution of nitrogen in caseinogen and in lactoglobulin shows some similarity as will be seen from the figures given by Crowther and Raistrick [1916].

It was felt that phosphorylation of serum globulin would be of interest not only in so far as it helped to throw light upon the manner in which the synthetically introduced phosphorus atoms are held in combination with the protein but also with reference to the problem of the origin of caseinogen in

the mammary cell. The stability of the phosphorus in phosphorised globulin towards acids, alkalis and enzyme preparations has been investigated upon lines similar to those adopted by Rimington and Kay in the study of caseinogen, and a striking similarity has been found to exist between these two proteins, suggesting a similar type of linkage. It is hoped that the accumulation of additional facts will afford evidence, in the light of which the hypothesis outlined above may be examined and a clearer knowledge obtained of the materials entering into the synthesis of caseinogen *in vivo*.

Phosphorylation of proteins.

In all cases the proteins were first thoroughly purified by solution and reprecipitation at the isoelectric point several times in succession. Caseinogen was directly prepared from separated milk. Care was taken to avoid undue exposure to alkali, the sodium hydroxide being added drop by drop, over a period of several hours to the suspension of protein in water which was vigorously agitated by a mechanical stirrer. A quantity of the protein, between 7 and 10 g., was then dissolved in water with the aid of the requisite quantity of sodium hydroxide, the total volume being brought up to 125–150 cc.

Phosphorus oxychloride (20–25 g.) dissolved in eight times its volume of carbon tetrachloride was allowed to drop into the ice-cold, rapidly stirred protein solution, to which had been added phenolphthalein to serve as an indicator, and the reaction was kept faintly alkaline by addition of 4*N* sodium hydroxide. The addition of the oxychloride extended over 6–8 hours and the temperature of the reactants never rose above 5°.

After separating the aqueous layer from the carbon tetrachloride, the phosphorised protein could be precipitated by addition of 2*N* hydrochloric acid until maximum separation occurred. This corresponded to a p_H between 3 and 4. The filtrate was kept for nitrogen determination and the precipitate purified as outlined above by dissolving in 0·1*N* sodium hydroxide and reprecipitating by hydrochloric acid, the filtrates from each reprecipitation being analysed for nitrogen and phosphorus. The data thus obtained served for the calculation of the P/*N* ratios (see Table I) and when this became constant the product was dehydrated by means of alcohol, dried over sulphuric acid *in vacuo* and a portion taken for analysis.

Phosphorised caseinogen.

In view of the fact that evidence has been obtained by Rimington and Kay [1926] suggesting the existence of an ester linkage in caseinogen between phosphoric acid and some other constituent of the molecule, it became of interest to find out whether by artificial means still further quantities of phosphorus could be induced to combine with the protein and, if this took place, to ascertain if possible the nature of the combination.

A quantity of 18 g. was dissolved in sufficient sodium hydroxide to give a neutral solution and the volume made up to 250 cc. Using the precautions described above, 50 g. of phosphorus oxychloride dissolved in 200 cc. of carbon tetrachloride were then added to the ice-cold solution in such a way that the operation was completed in 9 hours, sodium hydroxide solution being added as required to maintain a faintly alkaline reaction.

The phosphorised product was precipitated by addition of 59·5 cc. of 2*N* hydrochloric acid, filtered off, washed with a litre of distilled water and redissolved in the minimum quantity (87 cc.) of 0·2*N* sodium hydroxide. The exact equivalent of hydrochloric acid (0·2*N*) was then added very slowly to the rapidly stirred solution and the process of filtration, washing, etc., repeated until purification was complete as shown by the constancy of the P/*N* ratio in the filtrate from the precipitated protein. As a rule, five or six reprecipitations were found necessary before this stage was reached.

Table I.

Purification of phosphorised caseinogen. Nitrogen and phosphorus contents of the filtrates from each reprecipitation.

Filtrate	Volume cc.	Total nitrogen mg.	Total phosphorus mg.	P/N
1	300	31·2	—	—
2	242	4·55	5·79	1·27
3	320	6·53	2·83	0·43
4	248	6·35	1·24	0·20
5	266	5·43	1·21	0·22
6	300	3·83	0·60	0·16

The product was finally dehydrated by alcohol, and dried over sulphuric acid *in vacuo*. The yield was 61 %. Upon analysis it gave the following results, from which it will be seen that a further 0·97 % of phosphorus had been introduced into the molecule.

Analysis

H_2O lost at 107° (% of wet weight)	7·72
Total N (Kjeldahl, % of dry weight)	13·53
Total P (Neumann, % of dry weight)	1·77

Ratio P/*N* = 0·130. A second preparation gave P/*N* = 0·131.

The substance in all appearances resembles caseinogen but requires nearly twice as much alkali to bring it into solution.

It was shown by Rimington and Kay [1926] that whereas the phosphorus of caseinogen was quite stable to 0·25*N* hydrochloric acid at 37° and to bone phosphatase preparations, the action of 0·25*N* sodium hydroxide at 37° brought about the liberation of the whole of the phosphorus as inorganic sodium phosphate in 24 hours. Phosphorised caseinogen when tested in the same way gave the following results:

Trypsin	Slow liberation of inorganic phosphorus.
Pepsin	Very slow liberation of inorganic phosphorus.
Bone phosphatase ...	No liberation of inorganic phosphorus.
0·25*N* hydrochloric acid	Very slow liberation of inorganic phosphorus. Slightly slower than with pepsin.
0·25*N* sodium hydroxide	Complete liberation of phosphorus as phosphate in 24 hours.

Phosphorised caseinogen is also coagulated by rennin in the presence of calcium chloride, a fact which seems to show that whatever modification the treatment with phosphorus oxychloride may have produced, no radical structural change can have taken place, such as, for example, destructive hydrolytic cleavage.

Re-phosphorised caseinogen.

The name "dephosphorised caseinogen" was given by Rimington and Kay to the phosphorus-free protein obtained by allowing 1 % sodium hydroxide to act upon caseinogen for 24 hours at 37°. The substance so formed resembles caseinogen, but has an appreciably lower nitrogen content. An analysis of dephosphorised caseinogen by the Van Slyke N-distribution method (see Rimington [1927]) has shown that the loss has occurred chiefly in the amide nitrogen, but also to some extent in the arginine fraction, this amino-acid having presumably been partially converted into ornithine and ammonia.

Dephosphorised caseinogen is not coagulated by rennin (one sample did, however, show slight coagulation after many hours), but appears in all other respects to be very similar to caseinogen, except for less ready hydrolysis by proteolytic enzymes, due no doubt to partial racemisation having occurred during the action of the alkali.

4·7 g. of a preparation of dephosphorised caseinogen were phosphorised by means of 13 g. of phosphorus oxychloride in 75 cc. of carbon tetrachloride and yielded a substance, here referred to as "re-phosphorised caseinogen."

Analysis

Total N (Kjeldahl)	...	...	...	14·16 %	P/N = 0·124
Total P (Neumann)	...	...	...	1·75 %	

It was not appreciably acted on by proteolytic enzymes nor by bone phosphatase preparations, but lost all its phosphorus as phosphoric acid in 24 hours when acted upon by 0·25*N* sodium hydroxide at 37°. Towards 0·25*N* hydrochloric acid it displayed the same behaviour as phosphorised caseinogen.

Re-phosphorised caseinogen was found to be coagulated by rennin in the presence of calcium chloride, although less rapidly than is caseinogen under the same conditions. Possibly the property of coagulability by rennin is a feature dependent upon the presence in the molecule of organically bound phosphorus. That the nature of the protein is also of importance is what would have been expected *a priori*. Phosphorised globulin prepared by the phosphorylation of alcohol-denatured serum globulin yields insoluble precipitates at once on the addition of soluble calcium salts and one is led to wonder whether the obscure modification of physical properties brought about by the action of rennin upon caseinogen solutions is not, in reality, a process analogous to the denaturation of albumins and globulins by such agents as alcohol, heat, etc.

Phosphorised globulin.

All samples of protein used in this preparation were denaturated by addition to their aqueous solutions of 5 volumes of alcohol and 30 cc. per litre of a salt buffer mixture[1]. The protein was then purified by frequent reprecipitation, brought into solution in dilute sodium hydroxide and phosphorised in the usual way. It was found, however, that during the phosphorylation of globulin there is persistently formed a small quantity of material which is only soluble with difficulty in dilute acids and alkalis. This substance, unless removed by centrifugation, renders the subsequent operations involved in the purification of the phosphorised globulin somewhat difficult. It contains phosphorus in about the same proportion as phosphorised globulin but has not been further investigated.

The dried phosphorised globulin is a fine, white powder, insoluble in water, but dissolved by addition of sodium hydroxide. Its solutions give an immediate precipitate on addition of calcium chloride solution.

Analysis

H_2O lost at 107° (% wet weight)	10·19
Total N (Kjeldahl, % dry weight)	13·85
Total P (Neumann, % dry weight)	0·71
Ratio P/N = 0·051	

In order to investigate the stability of the combined phosphorus towards enzyme preparations, etc., solutions of phosphorised globulin were incubated at 37° with the different enzymes at the requisite p_H and in presence of suitable buffers. Specimens were taken at intervals, and inorganic phosphorus was determined colorimetrically by the method of Bell and Doisy.

The results, with the exception of the action of trypsin, are summarised below:

Bone phosphatase	No inorganic P formed.
Pepsin	Very slow liberation of inorganic P, 40 % as PO_4 in 10 days.
Bone phosphatase after pepsin...	No liberation of inorganic P.
0·25*N* hydrochloric acid ...	Very slow liberation of inorganic P, 35 % as PO_4 in 10 days.
0·25*N* sodium hydroxide ...	Complete liberation of P as PO_4 in 24 hours.

The action of trypsin on phosphorised globulin.

It was shown by Rimington and Kay that trypsin very rapidly liberates the whole of the phosphorus from caseinogen in the form of a diffusible, organic compound which is not precipitated by trichloroacetic acid. This substance is very slowly acted upon by trypsin with the formation of inorganic phosphoric acid, but is hydrolysed with extreme rapidity by bone phosphatase preparations, which split off approximately 67 % of the phosphorus as PO_4. The remaining 33 % may be liberated by means of kidney phosphatase

[1] Made up as follows:

Sol. A. 40 g. potassium sulphate, 202·5 g. sodium sulphate (cryst.), 360 cc. water heated up to effect solution then cooled.

Sol. B. Normal acetic acid.

Sol. C. Normal sodium acetate.

Equal volumes of A, B and C are mixed: $p_H = 4·7–4·8$.

preparations. Neither kidney nor bone phosphatase, however, has any appreciable action upon the phosphorus of caseinogen itself.

Phosphorised globulin was investigated in a similar manner. It was found that, in this case also, *trypsin brought about a very rapid liberation of the entire phosphorus in the form of a soluble organic compound*, but only brought about the subsequent hydrolysis to free phosphoric acid at an extremely slow rate. Bone phosphatase preparations, although they have, as just mentioned, no action upon phosphorised globulin, produced, when added to the solutions containing this organic, soluble derivative, *a liberation of the entire phosphorus as phosphoric acid within* 10 *hours*. This is shown in the accompanying curve, Fig. 1, the curve obtained from caseinogen using the same enzyme preparations being shown for comparison in Fig. 2.

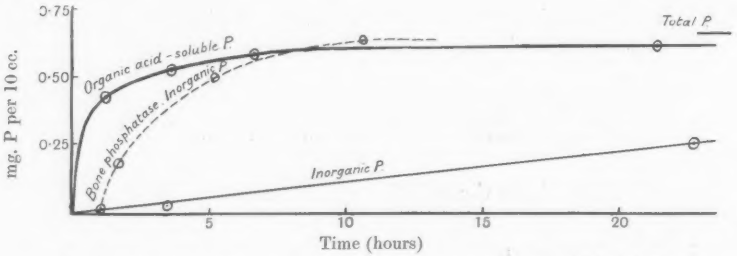

Fig. 1. Action of trypsin and of bone phosphatase on phosphorised globulin.
——— trypsin. – – – – bone phosphatase.

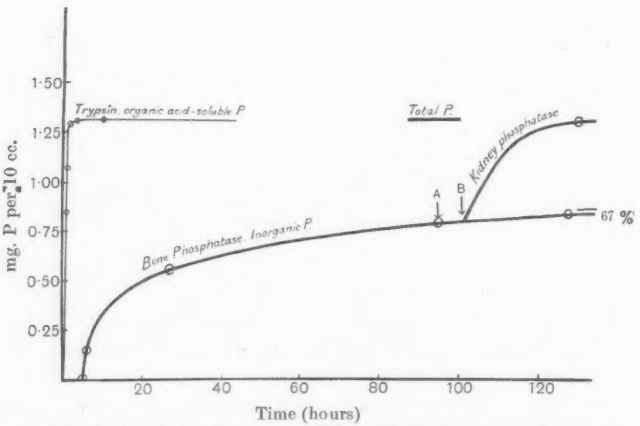

Fig. 2. Action of trypsin, bone and kidney phosphatase on caseinogen.
At A a further quantity of bone phosphatase was added.
At B kidney phosphatase (rabbit) was added.

It would appear therefore that a similarity exists between the nature of the phosphorus linkage in phosphorised globulin and in caseinogen, with the exception that, in the latter, a portion (1/3rd) of the total phosphorus differs from the remainder in being stable towards bone phosphatase, although hydrolysed by kidney phosphatase preparations.

The nature of the linkage involved in the phosphorus-containing proteins.

While as yet no decisive evidence is available, the work already published would render it highly probable that in caseinogen phosphorus is held, in part at least, by means of an ester linkage between phosphoric acid and some constituent of the caseinogen molecule. The existence of a possible NH-P linkage between an amino-group and phosphoric acid seems to have been disproved by the experiments of Rimington and Kay [1926], and evidence is also presented here against the view that such a combination takes place when proteins are phosphorised by phosphorus oxychloride[1].

Should such a phosphorus-nitrogen linkage exist, it should, on hydrolysis, give rise to a free amino-group titratable by means of formaldehyde according to the Sørensen technique. In order to test this possibility, suitable quantities of the phosphorised proteins were incubated at 37° with 0·25*N* sodium hydroxide for 24 hours under which conditions the whole of the phosphorus is removed as phosphate. The solutions were then aerated for two or three hours at room temperature in order to remove any ammonia [see Rimington and Kay, 1926] and the amino-nitrogen was determined by formaldehyde titration, a sample of the mixture having been titrated immediately after the addition of the sodium hydroxide. In no case was any increase in titratable groups found which was outside the error of the method. This fact in conjunction with the ready hydrolysis by the phosphoric esterase of bone would indicate that the phosphorus has entered the molecule in combination with some hydroxyl group.

Now the number of free hydroxyl groups in proteins is not known, although within the past few years several hydroxy-derivatives of previously known amino-acids have been found to be integral constituents of the protein molecule. Thus Dakin [1918] has estimated the amount of hydroxyglutamic acid in caseinogen as 10 % so that it is probable that the number of free hydroxyl groups is sufficient to account for the quantities of phosphorus which proteins are found capable of taking up. The method might even serve as some guide to the number of free hydroxyl groups present.

The possibility must be borne in mind, however, of the existence in the protein molecule of diketopiperazine rings which, in the "enol" form would be capable of taking up phosphorus just as the hydroxyl group of an amino-

[1] *p*-Chloroanilinephosphinic acid—in which phosphorus is directly linked to nitrogen—was found to be comparatively stable in alkaline solution but was decomposed rapidly with liberation of phosphoric acid by 0·25*N* hydrochloric acid at 37°. Neither trypsin nor phosphatases had any action on this substance.

acid. Whether these cyclic structures do exist, as maintained by Abderhalden and Komm [1924], in natural proteins, and if so in what form, are questions which are as yet undecided, but even supposing an equilibrium to exist between "keto" and "enol" modification, it is quite conceivable that phosphorus oxychloride, by combining with the "enol" form might so alter the equilibrium that eventually all the diketopiperazines pass over into this type. In this connection it is of interest to recall the stability of the diketopiperazine ring to trypsin and to compare it with the resistance offered by the organic, soluble phosphorus compounds of caseinogen and of phosphorised globulin towards this enzyme.

According to Bergmann [1924], a certain lability characterises the derivatives of the amino-acid serine, in which a benzoyl group has been introduced in place of hydrogen. Bergmann found that so slight a change as that of the medium from acid to alkaline reaction was sufficient to cause migration of the benzoyl group from union with the hydroxyl to the nitrogen atom.

As a result of his investigations, he is led to the view that such interchanges may take place in the protein molecule, thus furnishing a possible explanation of the extreme lability characteristic of protein material. One cannot discount the possibility that a phosphoryl group might behave in a manner at any rate analogous to the benzoyl group, although, up to the present, no experimental fact has been encountered supporting such an opinion.

Summary.

1. Proteins may be phosphorised by means of phosphorus oxychloride in a manner analogous to the Schotten-Baumann reaction.

2. Caseinogen, serum globulin and dephosphorised caseinogen have been treated in this way. Phosphorised caseinogen contains 1·77 % P, corresponding to a ratio P/N = 0·130. "Re-phosphorised caseinogen," the product obtained from dephosphorised caseinogen, contains 1·75 % P and has a ratio P/N = 0·124. Both substances are coagulable by rennin.

3. Phosphorised globulin contains 0·71 % P with P/N = 0·051. It is precipitated by soluble calcium salts.

4. The phosphorised proteins lose phosphorus slowly as phosphoric acid under the action of pepsin or 0·25 *N* hydrochloric acid, but 0·25 *N* sodium hydroxide at 37° liberates the whole of the phosphorus as phosphoric acid in 24 hours. Bone phosphatase liberates *no* inorganic phosphorus.

5. Trypsin brings about a rapid separation of the whole of the phosphorus of phosphorised globulin in the form of an organic, acid-soluble substance. It only liberates phosphoric acid from the compound with extreme slowness.

6. Bone phosphatase liberates the whole of the phosphorus from this organic substance as phosphoric acid within a few hours. In this respect, phosphorised globulin exhibits a similarity to caseinogen.

7. Phosphorus appears to enter the protein by means of an ester linkage between phosphoric acid and some hydroxyl group.

In conclusion I wish to thank Professor S. P. L. Sørensen of the Carlsberg Laboratorium, Copenhagen, for the great kindness and hospitality shown to me when working in his laboratory where the greater part of this work was done and for much helpful criticism and advice received from him. My thanks are also due to Sir F. G. Hopkins for the interest he has taken in the latter part of the work.

I am indebted to the Department of Scientific and Industrial Research for an allowance towards travelling expenses.

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XL. THE DIETARY VALUE OF POTATO PROTEIN.

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(*Received January 14th, 1927.*)

In a previous paper [Hartwell, 1925] it was shown that rats fed on a diet of potato, butter and salt mixture grew slowly and exhibited loss of fur. It was suggested that the use of new potatoes instead of old ones might lead to better results, and therefore further experiments on the same lines were started. Some years previously McCollum, Simmonds and Parsons [1918] fed young rats on potato supplemented in various ways. The potato used by these workers was dried, and ground up, so that the experiments are not strictly comparable with those described in this paper, where the object was to feed the potato in a form in which it is taken by man. McCollum and his co-workers found that the potato was poor in calcium, sodium and chlorine; therefore to guard against a shortage of any mineral constituents, salt mixture [Hartwell, 1922] was used in the diet described in this paper. Potatoes appear to contain quite large quantities of vitamin B [Steenbock and Gross, 1919] and Osborne and Mendel [1920, 1] find that there is no appreciable difference between old and new potatoes in respect of this factor. With regard to vitamin A, there appears to be some difference of opinion. Steenbock and Gross [1919] found that one lot of potatoes contained sufficient fat-soluble vitamin to support growth in young rats, while another sample contained none. This discrepancy in results must surely be due to some difference in technique not realised in this earlier work. Osborne and Mendel [1920, 2] find that the potato as a whole cannot be entirely devoid of fat-soluble vitamins. It seemed, therefore, safer to give butter fat in the diet and thus to guard against any possible deficiency. Incidentally the fat was useful for increasing the calorie value of the food.

Experimental.

Diet. The diet consisted of potato, butter, salt mixture and water. The potatoes were boiled in their skins and then peeled and mashed. The proportions used were 100 g. boiled potato, 1 g. salt mixture, 5 g. butter and 25 cc. of water to make a stiff paste. Food was given *ad lib.* and distilled water was provided in the usual water bottles.

Exp. 1. *Effect of the potato diet during growth.* 12 young rats, 6♀ + 6♂, were given the potato diet from weaning (3 weeks and 2 days). The experiment lasted just over 3 months, from April 14 to July 21, 1924. Old potatoes were used. The average ♂ curve is given in a previous paper [Hartwell, 1925].

Exp. 2. *Effect of the potato diet during growth, gestation and lactation.* This experiment was the same as Exp. 1, except that new potatoes were used instead of old ones and the feeding was continued for a longer period (August 18, 1924–May 21, 1925).

Exp. 3. *Effect of the potato diet during lactation only.* Six stock does were fed on a mixed diet of kitchen scraps and bread and milk until litters were born, after which they were given the potato diet.

Exp. 4. *Effect of the potato diet supplemented with bread, during lactation only.* Six stock does similar to those used in Exp. 3 were given a diet consisting of 15 g. potato diet, 15 g. bread and 21 cc. water mixed well together. The feeding was started as soon as possible after the birth of the litter.

Method. In Exps. 1 and 2 the method used was that described in a former paper [Hartwell, 1926]. For Exps. 3 and 4 the technique has been fully described in earlier papers on mammary secretion.

Results and Discussion.

Growth. In Exps. 1 and 2 most of the young rats grew, but very slowly compared with control animals. Two of the males showed maintenance of weight, but hardly any growth. Their condition was poor, and their fur came out badly [Hartwell, 1925]. It eventually grew again, but the coats were always thin. Rats fed on new potatoes appeared no better than those fed on old ones. In Exp. 2 the animals had an old look at a comparatively young age. That this frequently occurs when rats are fed on a poor diet has been shown by McCollum, Simmonds and Parsons [1918]. The young animals were particularly susceptible to cold, and, as shown in Fig. 1, several of them died after a short period on the potato diet. *Post mortem* the lungs were found to be badly affected in all cases. McCollum, Simmonds and Parsons [1918] fed rats on diets consisting largely of potato, and state that "growth took place slowly or not at all, individual animals shewing somewhat different capacity to make use of the diet." The results described here are quite in agreement with this statement. These observers gave 7–8 % of potato protein in the food (using dried potato), and the diet used by me could not have contained more than 7·6 % of protein. A possible explanation of the failure of growth in some animals is the low protein content, which may be borderline, and hence some of the rats were unable to adjust their metabolism to this low level of intake. That the rats were in fairly good health is shown by the rapid growth and improvement in condition produced by changing the diet. In Exp. 1 the food was changed to a mixture of 15 g. bread, 6 g. food casein [Hartwell, 1922], 0·4 g. salt mixture, 1·5 g. butter and 45 cc. of 3 % marmite.

After 10 days the animals were in good condition; the fur was thick and glossy and they had grown much more rapidly.

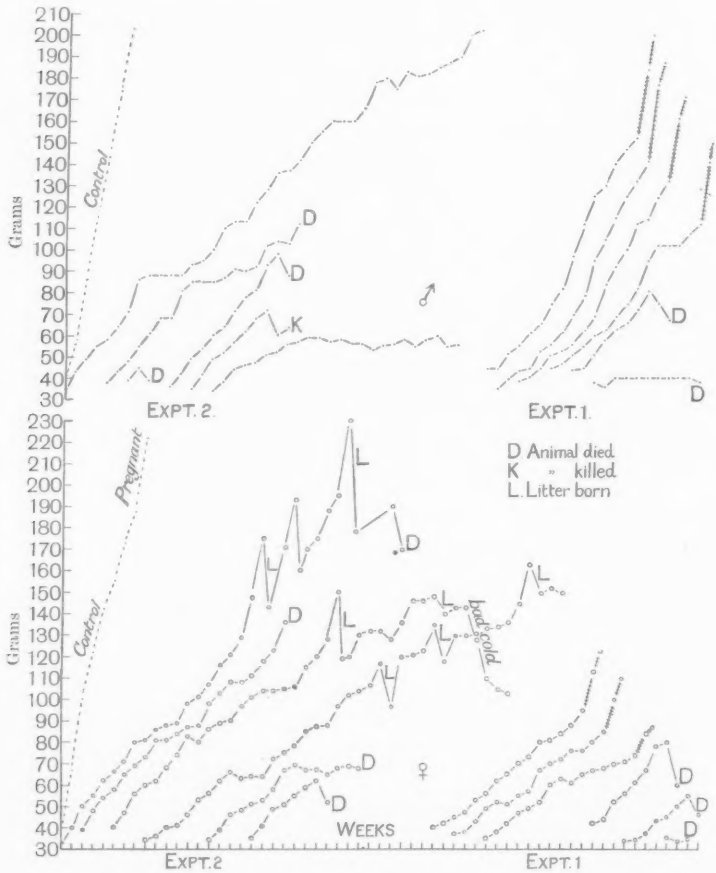

Fig. 1. Growth curves of young rats fed on a diet of potato, butter, salt mixture and water.
----- Diet changed to bread, food casein, salt mixture, butter and marmite.

Maintenance. No experiments were made to test the efficiency of potato protein as regards maintenance, but it seems reasonable to suppose that a diet which provides for growth (and reproduction), at any rate in some animals, is adequate for maintenance. Rose and Cooper [1917] found that a woman was able to maintain nitrogenous equilibrium for 7 days on a diet of potato, sugar, and agar-agar; they conclude, therefore, that the nitrogenous compounds of the potato possess a high nutritive efficiency.

Hindhede [1913] considers that "vegetable foodstuffs, and potatoes in particular, are considerably better digested than animal." He calculates (from the experiments of Constantinidi on man) that of 3000 calories in potatoes and fat, only 2 % go to waste in the faeces, while in meat and fat, or milk, the loss is 5 %.

Reproduction. No young were born until the does were about $5\frac{1}{2}$ months old, while stock animals produce and rear their young as early as 3 months. An explanation of this probably lies in the fact that the rats on the potato diet were very small for their age and of poor weight. The breeding results were as follows:

Rat 1[1]. Produced 3 litters.
1. 3+1 dead—all eaten 11th day.
2. 1+7 dead—dead 6th day.
3. 7+4 dead—3 reared—average weight at 21 days 36·3 g.

Rat 2[1]. Produced 3 litters.
1. 1+3 dead—dead 2nd day.
2. Born dead.
3. Born dead.

Rat 3. Died before any young born.

Rat 4. Produced 2 litters.
1. 3—eaten 3rd day.
2. Eaten 2nd day.

Rat 5. Died before any young born.

Rat 6. Died at birth of 1st litter.

The rats fed exclusively on the potato diet were not sterile, but the food must be regarded as very unsatisfactory for gestation, because so many of the young were born dead. The noticeable failure to rear the young (even when a good diet, such as bread and milk, was given to the mother rat after their birth) suggests that those which were alive were weakly. All the young were of low weight at birth. The low percentage of protein in the diet may account for these poor results.

Lactation. Exp. 2 gave no results in this respect; only one litter was reared and the mother had been given bread and milk.

Exp. 3 showed that it was possible for the rat to rear her young when she was fed on the potato diet during lactation. Each rat was allowed to keep six of her babies, but only one animal succeeded in bringing up the whole litter. The growth curve of these is shown in Fig. 2. The other rats lost two or three of their young at different stages of lactation. From Fig. 2 it is obvious that the mother rat lost weight during lactation. This loss of weight occurred in all cases. The young grew slowly, and were below normal weight at weaning; their fur was thin and poor. The general condition of the young was similar to that obtained when the mother rat was fed on a diet of bread, butter and salt mixture, but with the bread diet the weight curve of the young was slightly better, though the mother rat lost weight as badly. (1182 in Fig. 2 is put in for comparison.)

[1] Bread and milk diet given after the birth of the litter.

In Exp. 4 bread was added to the potato diet, and, as seen from the figure, the growth of the young rats was improved. Their general condition also was better, but they were still far below average weight, being only 23–24 g. at weaning instead of approximately 40 g. The supplementing of the potato and bread proteins is in agreement with the work of McCollum, Simmonds and Parsons [1921].

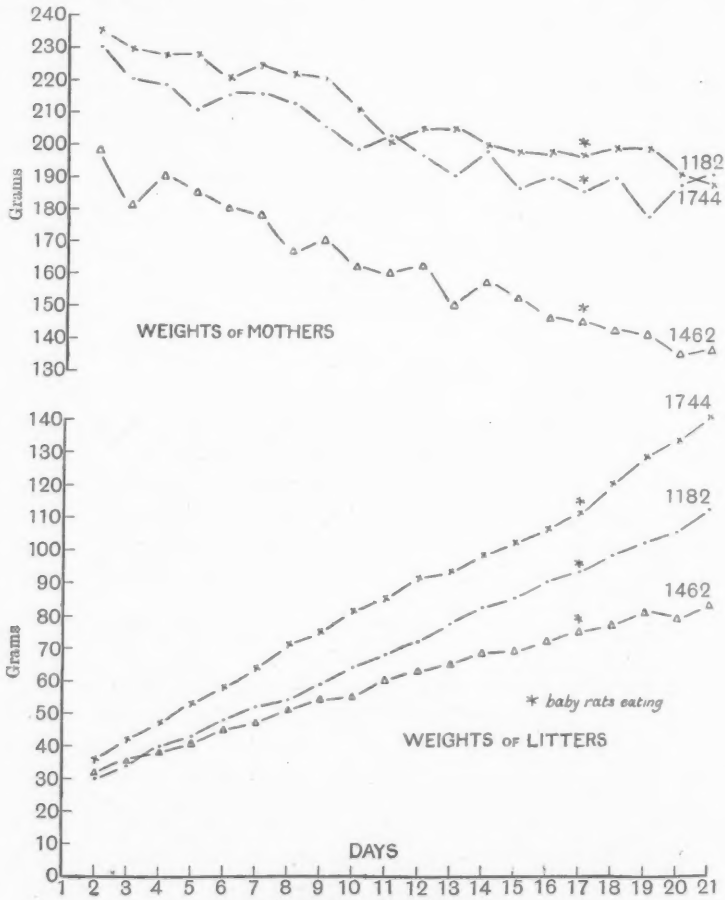

Fig. 2. Diets of mothers.

×——× 1744. Bread, potato diet and water.

•——• 1182. Bread, butter, salt mixture and water.

△——△ 1462. Potato, butter, salt mixture and water.

These experiments show that potato as the sole source of protein in the diet is not adequate to meet the needs of the rat. Is it the *quality* or the

quantity of the protein which is at fault? McCollum, Simmonds and Parsons [1918] consider that it is the *quality* which is the limiting factor. These workers used other diets "similar in every respect except that a portion of the carbohydrate was replaced by the purified protein casein." This modification led to rapid growth of the animals, but the author does not consider this a proof of any inadequacy in *quality*, because from the statement quoted above it is clear that a greater *quantity* of protein was used. In the experiments described in this paper, the percentage of protein in the food was only about 7·6, much too low to expect good growth. Therefore the fact that growth and even reproduction took place seems to suggest that the potato protein is of good quality and it is the insufficient amount which caused the poor results. In support of this hypothesis there is the work of Rose and Cooper [1917] and the observations of Hindhede [1913] previously quoted. Onslow [1920] states that potatoes contain a globulin, tuberin; such proteins are regarded as being of good quality. Further, tyrosine, lysine and leucine are said to be found in the tubers [Onslow, 1920]. According to Rose and Cooper [1917] only 63 % of the potato nitrogen is in the form of protein, but the animal organism is capable of using amino-acids, and tyrosine is an essential member of this group. A watery, protein-free extract of potato gives a well-marked Millon's reaction.

The calorie value of the potato diet was equivalent to that eaten by other rats giving normal growth curves.

Summary.

1. Potato protein provides for growth in some rats but the rate is very slow. Other rats fail to grow on the diet.

2. Reproduction takes place on a diet of potato, butter, salt mixture, and water, but many of the young are born dead, and hardly any of them are reared.

3. The rat can rear her young on the above diet (if she is given better food during gestation), but the babies are undersized and have poor coats; the mother rat loses weight during lactation.

4. The addition of bread to the potato diet during lactation results in improved growth and condition of the young rats, but the mother still loses weight.

5. It is suggested that the *quantity* rather than the *quality* of the potato protein is responsible for the slow growth.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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XLI. THE PHOSPHORUS NUCLEUS OF CASEINOGEN.

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(*Received January 21st, 1927.*)

In an interesting paper by Rimington and Kay [1926] entitled "The liberation of phosphorus from caseinogen by enzymes and other agents," I noticed the following sentence:

"We could find no account in the literature of any serious attempt to isolate the organic phosphorus compound or compounds produced during tryptic hydrolysis of caseinogen."

I think it my duty to call the attention of the authors to the fact that as early as 1923 the Society of Chemical Industry in Basle patented a process of mine for preparing the phosphorus nucleus of caseinogen by means of a short tryptic digestion (2–3 days) [1923, 1925].

As Rimington and Kay will easily realise, if they study the publication alluded to, I was able to isolate from the tryptic digestion liquor of caseinogen, with the aid of its lead salt, a phosphorus compound containing 5·86 % P and 11·90 % N, *i.e.* 4 atoms P for 18 atoms N.

The compound obtained by Rimington and Kay contains only 3·8–4·00 % P.

Later on, I succeeded in preparing more degraded polypeptides and determined their constitution. According to these researches the phosphorus nucleus of caseinogen is composed of four serine-phosphoric acids.

More details concerning the properties and physiological meaning of these phosphopolypeptides are to be found in my other publications [1926, 1927].

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XLII. CONTRIBUTIONS TO THE STUDY OF LIGNIN.

PART I. METALIGNIN, A NEW TYPE OF ALKALI LIGNIN.

By CHARLES DORÉE and EUSTACE CECIL BARTON-WRIGHT.

From the Chemistry Department, Chelsea Polytechnic, S.W.

(*Received January 29th, 1927.*)

Although the soda process for the resolution of lignified material into cellulose, and soluble derivatives of lignin, is an important industrial process, very little evidence has been recorded as to the nature and composition of the lignin so obtained, and of the relation existing between this form of lignin and the lignosulphonic acids obtained in the bisulphite process.

Powell and Whittaker [1924, 1925], who investigated the lignins obtained from flax and a number of woods, including pine, spruce, ash, birch and poplar, employed a solution of caustic soda of 8–12 % concentration for six to ten hours at 140–160°. They considered that this process does not involve internal changes in the lignin molecule, and that the lignin so obtained is "probably closely related to lignin as it exists in the lignocellulose complex." The results of a large number of comparative experiments showed that the lignin isolated from flax, and from the various woods under the above conditions, is of constant composition, viz. $C_{45}H_{48}O_{16}$, and the authors concluded that plant lignin is derived from a basic substance which they term "lignol," $C_{41}H_{40}O_{16}$. Lignol contains nine hydroxyl groups and the authors assume that the lignins present in the various woody tissues differ only in the extent to which the hydroxyl groupings of the basic lignol are methylated. The lignin $C_{45}H_{48}O_{16}$ contains four methoxyl and five hydroxyl groupings and the product obtained from spruce-wood showed on analysis the composition C = 64·0, H = 5·5 %.

There is no doubt that the working conditions, the time and temperature and the concentration of alkali used influence the resulting product. Under the prolonged action of alkali at high temperatures internal changes, and probably oxidation of the lignin complex, take place [Fuchs, 1926]. The data obtained by Beckmann, Liesche and Lehmann [1923] illustrate this variability in composition.

Beckmann, Liesche and Lehmann [1921] have employed perhaps the most restrained conditions in isolating lignin fractions from rye-straw; namely, 2 % sodium hydroxide in aqueous-alcoholic solution acting in the cold for

48 hours. The alcohol prevented the solution of hemicelluloses, pentosans and other non-lignified substances, which are soluble in, and removed by, aqueous alkalis.

Their product was a yellow powder which darkened and aggregated at 50°. Its molecular weight was of the order of 800 and its composition approximated to the formula, $C_{40}H_{44}O_{15}$, this unit containing 4 hydroxyl and 4 methoxyl groupings.

The two investigations cited illustrate extreme variations in working conditions. Other products obtained from commercial alkaline black liquors have been described [see Hägglund, 1924; Holmberg, 1921].

Apart from oxidation and definite internal change brought about by the prolonged action of caustic alkali, removal of one or more methoxyl groupings of the original lignin is probable, resulting in the isolation either of a single derivative, or of a mixture of partly demethylated derivatives. In order to obtain a lignin as near as possible to its original condition, it is desirable, therefore, to reduce the severity of the alkaline attack and shorten the time as far as consistent with satisfactory resolution.

In the course of experiments having for their object the quantitative separation of lignin from lignified tissues, Mehta [1925] found that sodium hydroxide solution in a concentration of 4 % gave the best result, and that with this strength of solution at a pressure of 10 atmospheres the resolution of the wood was complete in one hour. The lignin is stated to be isolated quantitatively on acidifying the solution.

The method was used by this author for the estimation of lignin in a large number of plant products—the values obtained being very much lower than those generally found by the hydrochloric acid method of Willstätter and Zechmeister [1913]. The lignin obtained from different species of wood was found to be a brown powder with the odour of vanillin, insoluble in water, but soluble in dilute alkalis, ammonia, alcohol, acetone and glacial acetic acid. It differed from all lignins previously described in that it had a fairly definite melting point (170°). The acid value was found to be 477 and the iodine value 139·7.

The method appeared to us likely to yield a lignin with the minimum of chemical change, while the marked solubility in a range of organic solvents and the evidence of a definite melting point seemed to indicate a lignin of relatively simple composition, in agreement with our own results on jute fibre. By arrangement with Dr D. N. Nanji, therefore, we have carried out an investigation of the substance, the results of which are given below.

It has been overlooked by most previous workers that caustic alkali removes from the wood not only lignin, but also resins, fat-waxes and hemicelluloses which it may be impossible to remove subsequently from the lignin and this may very well account for the variable compositions reported by different workers. The wood used throughout this investigation was spruce, which was thoroughly extracted with benzene, alcohol and water and then digested

with 5 % sodium hydroxide solution in the cold to remove hemicellulose. The lignin isolated by us was a brown powder, soluble in most organic solvents with the exception of ether, benzene and light petroleum. Its melting point was 186°. It was soluble in sodium carbonate, but not in ammonium carbonate solutions. It had the composition $C_{20}H_{20}O_6$, and determinations of the molecular weight gave the somewhat surprising result that this formula represented the molecular unit[1]. We have thus, as anticipated, a compound of relatively simple composition; almost all lignins previously isolated having been assigned formulae with approximately double this number of carbon atoms. As the product is possibly a depolymerised form of the lignin as it exists in the wood and as it exhibits definite properties by which it can be identified, we propose to refer to it as *metalignin*.

Metalignin, $C_{20}H_{20}O_6$, has one free hydroxyl group, two methoxyl groups and, according to the composition of the oxime it contains, two carbonyl groups, one of which may be ketonic and the other aldehydic. The iodine value of 139·7, previously recorded, corresponds to two unsaturated linkages in the molecule (found: $C_{20}H_{20}O_6$ of molecular weight 356 absorbs iodine 498·4, $2I_2$ requires 508). It is interesting to note in this connection, that Cross and Bevan [1882] isolated a small alcohol-soluble fraction from chlorinated jute which had the composition $C_{19}H_{18}O_9Cl_4$ with one free hydroxyl grouping. These results together with those recorded for the alkali lignins are summed up in the following schematic formulae:

$C_{16}H_{12}O\begin{cases}OH\\(OMe)_2\\CO\\CHO\end{cases}$ $\qquad$ $C_{36}H_{28}O_{11}\begin{cases}(OH)_4\\(OMe)_4\end{cases}$ $\qquad$ $C_{39}H_{30}O_4\begin{cases}(OH)_5\\(OMe)_4\\(CO)_2\\CHO\end{cases}$

I. Metalignin $C_{20}H_{20}O_6$ $\qquad$ II. Beckmann, Liesche and Lehmann: alkali lignin from rye-straw $C_{40}H_{44}O_{15}$ $\qquad$ III. Powell and Whittaker: lignin $C_{15}H_{48}O_{16}$

Preparation of metalignin.

Spruce-wood was employed throughout the present work as its constants are well known and the majority of the lignin derivatives previously described in the form of sulphonic acids were prepared from this wood. An analysis of the wood employed will be found on p. 299, from which it will be seen that it contains 5·8 % of extractives and 3·8 % of hemicelluloses. The purification of the wood followed generally on the lines recommended by Friedrich and Diwald [1925].

The wood, in the form of fine sawdust, was well extracted with benzene (12 hours), alcohol (12 hours) and water (12 hours) to remove resins and glucosides, and then digested with four changes of a 5 % solution of sodium hydroxide for 36 hours to remove hemicelluloses (for quantitative results see p. 299). The metalignin itself was isolated by heating the purified sawdust in an autoclave with a 4 % sodium hydroxide solution at 8 atmospheres

[1] It is interesting to note that Holmberg [1921], by the extraction of sulphite liquor with ether, has isolated a crystalline substance, M.P. 250°, of the same formula $C_{20}H_{20}O_6$, which is probably the lactone of diguaiacyltetramethylenecarbinolcarboxylic acid.

pressure for one hour[1]. The dark solution of sodium metalignate was poured off and the metalignin precipitated by the addition of hydrochloric acid, washed by decantation, dissolved in 95 % alcohol and the alcoholic solution evaporated to dryness. The metalignin thus obtained was a lustrous black solid.

It was purified by solution in glacial acetic acid and precipitation with water; the precipitated product was washed with water and dried at 60°. After the first precipitation the original value for the melting point, 170°, was raised to 180°, whilst after the second and third treatment the melting point remained constant at 185–186°.

Properties of metalignin.

The purified metalignin was a light brown, odourless solid, easily soluble in pyridine, alcohol, acetone, glacial acetic acid, ethyl acetate, naphthalene, trichloroacetic acid, saturated calcium thiocyanate solution and slightly soluble in chloroform. It was insoluble in ether, benzene, ligroin, light petroleum, as well as in sodium bisulphite solution, sulphurous acid and ammonium oxalate solution. It dissolved readily in sodium hydroxide, ammonium hydroxide and sodium carbonate solutions and was precipitated from these on the addition of acids. It was insoluble in ammonium carbonate solution. The calcium and barium salts were precipitated on the addition of the soluble salts of these elements to a solution of metalignin in ammonia. In its solubility in glacial acetic acid and acetone, it differed from the product obtained by Powell and Whittaker. The dry substance contained 0·13 % ash. Analyses are calculated on ash-free material.

Found: C = 66·8, H = 5·6; C = 67·0, H = 5·6; $C_{20}H_{20}O_6$ requires C = 67·3, H = 5·6.

The formula $C_{20}H_{20}O_6$ for metalignin is the same as that predicted by Klason for α-lignin (see below). Determinations of the molecular weight by the cryoscopic method in glacial acetic acid gave the average value 341.

0·4410 g. in 31·3 g. glacial acetic acid	$\Delta = 0{\cdot}164°$	M.W. =	336
0·8770 ,, ,,	$= 0{\cdot}314°$	,, =	341
1·3186 ,, ,,	$= 0{\cdot}473°$	,, =	347

The molecular weight was also determined in naphthalene and gave the average value 342.

The molecular formula is therefore equal to $C_{20}H_{20}O_6$. Two methoxyl groups were found to be present.

Found: OMe = 18·6 % and 17·4 %; $C_{18}H_{14}O_4(OCH_3)_2$ requires OMe = 17·4 %.

Monobenzoyl-metalignin $C_{20}H_{19}O_6(CO.C_6H_5)$. Metalignin reacted with benzoyl chloride in alkaline solution with great ease.

The monobenzoyl-metalignin was a pale yellow, almost white solid, M.P. 184°. It was very soluble in pyridine, less so in alcohol and insoluble

[1] A 10 % solution of sodium hydroxide at 4 atmospheres pressure for 1 hour also gave good results.

in ether, benzene, ligroin and light petroleum. The crude benzoyl derivative was dissolved in pyridine and precipitated with 95 % alcohol. After three such treatments the melting point became constant at 184°.

Found: C = 70·1 %, H = 5·1 %, OMe = 13·8 %; $C_{27}H_{24}O_7$ requires C = 70·4 %, H = 5·2 %, OMe = 13·4 %.

Monoacetyl-metalignin $C_{20}H_{19}O_6$ $(CO.CH_3)$. The acetyl derivative was prepared according to the description given by Powell and Whittaker [1924]. 5 g. metalignin and 20 g. acetyl chloride were mixed and a few drops of concentrated sulphuric acid added. The metalignin rapidly went into solution with evolution of a considerable amount of heat, the mixture being heated for one hour at 80°. The acetyl derivative was precipitated by pouring the hot solution into ice-water with vigorous stirring. The acetyl-metalignin was very soluble in pyridine, alcohol and acetone, insoluble in ether, benzene, ligroin and light petroleum. It was purified by dissolving in acetone and precipitating with brine, washing with water and drying at 60°. Unlike the acetyl derivative described by Powell and Whittaker it was quite stable.

Found: C = 66·0 %, H = 5·5 %; $C_{22}H_{22}O_7$ requires C = 66·3 %, H = 5·5 %.

Methyl-metalignin $C_{18}H_{13}O_3(OCH_3)_3$. 5 g. metalignin were dissolved in 12 cc. of 10 % caustic soda solution and dimethyl sulphate was added drop by drop with constant shaking. The methylated derivative was slowly precipitated. Purification was effected by dissolving the crude product in pyridine and precipitating with 45 % alcohol. The methylated derivative was a pale, almost canary yellow compound, very soluble in pyridine, acetone and alcohol, insoluble in ether, benzene and light petroleum.

Found: OMe = 25·4 %; $C_{18}H_{13}O_3(OCH_3)_3$ requires OMe = 25·1 %.

Metalignin dioxime $C_{20}H_{22}O_6N_2$. The metalignin contains active carbonyl groupings, which was shown by the fact that it reduced Fehling's solution and ammoniacal silver nitrate solution and by the preparation of the dioxime. This compound was prepared by the method described by Brady and Dunn [1913]. 3 g. metalignin were mixed with 3 g. hydroxylamine hydrochloride and an 8 % solution of sodium hydroxide was added gradually to the mixture. The solution was filtered and the oxime precipitated by the addition of ammonium chloride solution.

The oxime was a pale brown solid, soluble in pyridine and acetone, but only slightly soluble in alcohol. It was insoluble in ether, light petroleum and benzene. It was purified from alcohol solution.

Found: N = 6·7 % and 6·9 %; $C_{20}H_{22}O_6N_2$ requires N = 7·2 %.

Metalignin-phenylhydrazone. The preparation of a phenylhydrazine derivative proved unsatisfactory. The metalignin did not react with the phenylhydrazine at all readily, and it seemed doubtful if the compound finally obtained was the true hydrazone. With the exception, perhaps, of the compound obtained by Dorée and Hall [1924] from their lignosulphonic acid, the phenylhydrazones obtained from the various lignins do not stand in any definite relationship to the parent substance.

From the results recorded above, the formula

$$C_{16}H_{12}O(OCH_3)_2OH.CO.CHO$$

is given to metalignin. It will be seen that all the oxygen atoms, except one, are accounted for, leaving a nucleus of 16 carbon atoms.

It was hoped that on oxidation some definitely recognisable cleavage product would result. This was not the case.

Oxidation of metalignin.

(*a*) 5 g. metalignin were heated with 100 cc. of 5 % nitric acid on the water-bath for six hours, when an insoluble orange-red compound slowly formed, which proved to be soluble in all the solvents for metalignin itself. From its general reactions it appeared to be similar to the compound $C_{25}H_{27}N_2O_{17}.OCH_3$, isolated by Dorée and Hall [1924] by the oxidation of lignosulphonic acid with 5 % nitric acid. It gave ammonia on warming with sodium hydroxide and could be reduced with zinc dust and acetic acid to a brown substance. It was more acidic in character than metalignin, dissolving readily in alkalis and ammonium carbonate, from which solutions it could be precipitated on the addition of acid.

(*b*) 6 g. metalignin oxidised for 4 hours with 10 % nitric acid gave 3 g. oxalic acid. Dorée and Hall also discovered that when lignosulphonic acid was oxidised with strong nitric acid, oxalic acid was obtained in considerable yield.

(*c*) Metalignin was oxidised with sulphuric acid in the presence of mercuric sulphate at 165°. By this method a minute amount of the mercury salt of an acid was obtained, which was very soluble in ether, alcohol and water, slightly soluble in benzene and insoluble in light petroleum. It was, however, obtained in too small a yield to be identified.

The relation between the lignins isolated by different methods and the original lignin of the plant.

In Table I are collected data relating to some of the better defined types of lignin that have been described. As these vary in the number of methoxyl groupings they contain and some are sulphonic acids and others acetyl derivatives, the empirical formula for the basic "lignol" has been calculated in each case for purposes of comparison. This "lignol" represents the hydroxylated compound, sulphonic, acetyl and methyl groupings being eliminated. A certain general similarity will be observed among all these lignols. The number of carbon atoms approximates to 36 and the number of carbon and hydrogen atoms present is approximately equal. The number of oxygen atoms varies and in this connection we have noticed that isolated lignin has a remarkable capacity for fixing oxygen (under the action, for example, of nitric acid or hydrogen peroxide) which may account for this irregularity.

The fact that, using a particular method of isolation, *e.g.* that of No. 7, identical products are obtained from different woods, flax, etc., seems fairly

strong evidence for the assumption that the same lignin substance is present throughout the plant world. According to Friedrich and Diwald [1925] it would appear that in the wood tissue it exists as a methylated derivative. By the action of 17 % hydrochloric acid in the cold they claim to have isolated a lignin without hydrolysis of hydroxyl groupings or subsequent condensation changes which take place under the action of the 40 % hydrochloric acid used by Willstätter.

Table I.

	Method of isolation*	Author	Empirical formula	No. of OH groups	No. of OMe groups	Corresponding basic "lignol"	Lignol % C	Lignol % H
1.	17 % HCl cold	Friedrich and Diwald [1925]	$C_{39}H_{48}O_{14}$	1	5	$C_{34}H_{38}O_{14}$	60·8	5·6
2.	3 vol. HCl (d. 1·18) and 1 vol. H_3PO_4 (d. 1·7)	Urban [1926]	$C_{43}H_{48}O_{16}$	5	4	$C_{39}H_{40}O_{16}$	61·2	5·2
3.	H_2SO_3 aq. at 110°	Dorée and Hall [1924]	$C_{26}H_{30}O_{12}S$	3 or 4	2	$C_{24}H_{24}O_9$	63·2	5·26
4.	Ditto	Klason [1925]	$C_{30}H_{32}O_{12}S$	3 (?)	2 (?)	$C_{28}H_{26}O_9$	66·1	5·1
5.	Calcium bisulphite liquor—computed	Klason α-lignin [1920]	$C_{22}H_{22}O_7$	1	2	$C_{18}H_{16}O_6$	65·85	4·84
6.	4 % NaOH at 10 atmos. pressure 1 hour	Dorée and Wright Metalignin	$C_{20}H_{20}O_6$	1	2	$C_{18}H_{16}O_6$	65·85	4·84
7.	8–12 % NaOH at 140–160° for 6–10 hours	Powell and Whittaker [1925]	$C_{45}H_{48}O_{16}$	5	4	$C_{41}H_{40}O_{16}$	62·4	5·07
8.	2 % alcoholic NaOH in cold on rye-straw	Beckmann, Liesche and Lehmann [1921]	$C_{40}H_{44}O_{15}$	4	4	$C_{36}H_{36}O_{15}$	61·01	5·08

* Spruce-wood or allied soft wood unless otherwise stated [Fuchs, 1926, p. 281].

As will be seen (No. 1), this lignin contains 5 methoxy-groupings, of which two are readily removed on saponification and are believed to be ester methoxyl, whilst the remaining three are ether methoxyl.

Metalignin (No. 6) is characterised by melting point and solubility range and it differs from the others in possessing the molecular formula corresponding to a C_{20} unit, the others approximating to a C_{40} unit complex. It is interesting to note that Klason had previously deduced that an α-lignin of the same formula constituted (in the form of its sulphonic acid) one component of the lignin isolated in the calcium bisulphite process. Later [1925], in order to explain the existence of the acid $C_{26}H_{30}O_{12}S$ (No. 3) which is obtained by the action of sulphurous acid solution at 110°, Klason has doubled the formula for α-lignin and considers that the acid C_{26} is a mixture of a mono- and a dibasic sulphonic acid $2C_{20}H_{20}O_6 . 2H_2SO_3$ and $2C_{20}H_{20}O_6 . H_2SO_3$ respectively, while in a later communication he states that the simplest acid obtainable by the action of sulphurous acid solution on wood has the composition

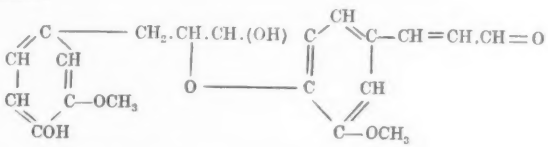
$$3C_{10}H_{10}O_3 . H_2SO_3.$$

There is thus a connection between the product obtained from spruce by sulphurous acid, and by the action of dilute alkali for a minimum time. It

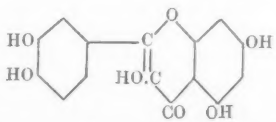
may be that metalignin, C_{20}, is the unit upon which the natural lignins are based and that, whilst the usual type isolated is of the order C_{40}, it may exist in the plant in an even more polymerised form. In this way its resistance to oxidation in the living tissue and its susceptibility when isolated may be explained.

With regard to the constitution of metalignin, we do not assume the presence of the acidic (carboxyl) grouping. The fully methylated derivative is insoluble in sodium hydroxide, so that the solubility in alkali hydroxides appears to be due to the one free (phenolic?) hydroxyl group. From the evidence of the oxime we assume the presence of one carbonyl-group and one aldehydo-group in agreement with the results of Powell and Whittaker, who postulated two carbonyl- and one aldehydo-groupings in the C_{45} unit complex. The extended formula thus becomes: $C_{16}H_{12}O(OCH_3)_2.OH.CO.CHO$.

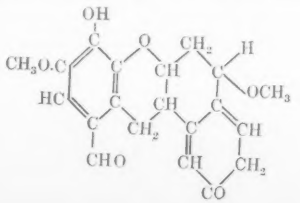
We have obtained a certain amount of evidence which makes it probable that the unassigned atom of oxygen exists in a pyrone ring and that the $CO\text{-}CH_2$ group is also present. It will be remembered that Klason regards α-lignin as derived from 2 molecules of coniferyl aldehyde, giving the following formula:



in which two aromatic nuclei are present. It seems impossible, with such a constitution, to explain the fact that on oxidation no aromatic products are obtained. The Klason formula closely resembles that of the anthocyanins, *e.g.* quercetin:



and the tetramethyl ether of this on oxidation with alkaline potassium permanganate yields veratric acid $C_6H_3(COOH)(CH_3)_2$, and probably phloroglucinol dimethyl ether. With metalignin on the other hand gentle oxidation, *e.g.* with dilute nitric acid, results in complete breakdown to oxalic and carbonic acids with the intermediate formation of non-aromatic nitrocompounds. We therefore prefer to regard metalignin as based upon hydroaromatic nuclei in some such way as shown in the following ideal formula:



in which the two double linkages and the benzo-pyrone ring are readily opened up on oxidation tending to the disruption of the entire complex.

Alkali resolution as a method for the estimation of lignin.

Mehta [1925] has developed the following process for the quantitative estimation of lignin in wood. 5 g. dry wood in the form of fine sawdust are treated with 100 cc. of 4 % sodium hydroxide solution at 10 atmospheres pressure in a Pyrex glass tube for one hour. The lignin is said to be removed quantitatively as a sodium derivative leaving a pure α-cellulose. The solution is acidified, and the precipitate dissolved in 95 % alcohol. The alcohol is removed and the residue dried to constant weight at 100°.

The figures arrived at, compared with those obtained on the same samples by the Willstätter process, show marked differences, as will be seen in Table II.

Table II.

	4 % NaOH	H_2SO_4.aq.	Difference
Lignum vitae ...	38·39	47·99	9·6 %
Teak	31·07	45·80	14·7
Larch	21·33	32·80	14·7
Spruce	17·80	30·55	12·7

The higher values obtained by the Willstätter method are ascribed to the presence of impurities and to combination with SO_4H residues. The presence of impurities to the extent of 14·7 % is certainly high, and it is doubtful if the discrepancy can be due to this cause.

In the present work a quantitative examination was made of the spruce sawdust employed, to see if the difference in the values obtained by the two methods could be accounted for in any way. About 20 g. spruce wood, after extraction with benzene and alcohol and 5 % sodium hydroxide, were heated with 4 % sodium hydroxide in an autoclave for one hour at 10 atmospheres pressure. The sodium lignate was filtered off and the residual "cellulose" examined. This was at first red in colour, but quickly oxidised in the air to brown and finally to a dull green. After washing and drying this colour was still retained. It was never "snow white" or "exceptionally light" in colour as described by Mehta. Treatment of this "cellulose" with hydrochloric acid (d. 1·2) for twelve hours showed that it contained 5·4 % of lignin.

An examination was also made of the filtrate after precipitation of the lignin with hydrochloric acid. This was of a pale yellow colour which deepened to brown on the addition of alkali. The filtrate did not give a precipitate with a solution of calcium chloride, a result at variance with that of Powell and Whittaker. After neutralisation with ammonia, the liquid was evaporated almost to dryness on the water-bath, when a dark solid was obtained which was removed by extraction with alcohol. The alcoholic extract gave a brown

residue, soluble in water, pyridine, alcohol, and alkalis. It had an odour of ω-hydroxy-methylfurfural.

A more exact quantitative experiment was also carried out: 5 g. spruce sawdust, after extraction for eight hours with benzene and alcohol and with water for four hours, were finally digested with a 5 % solution of sodium hydroxide for twelve hours to dissolve the hemicelluloses present. The purified sawdust was then autoclaved for one hour with 100 cc. of 4 % sodium hydroxide at 10 atmospheres pressure. The following values were obtained:

Metalignin (precipitated by acid)	18·6 %
Residue from filtrate (not precipitated by acid) ...	7·3
Lignin remaining in the cellulose	3·7
Total	29·6

In a further effort to ascertain the cause of the difference between the values obtained by Mehta's method and the hydrochloric acid method all the constituents of the wood were estimated by the methods of Dore [1920] (with the exception of the lignin). The following values were obtained (calculated on the original dry spruce sawdust, which contained 10 % of natural moisture):

Extraction with benzene	1·7 %
,, alcohol	1·6
,, water	2·5
,, sodium hydroxide (5 %) ...	3·8
Cellulose	55·0
Mannan	7·6
Galactan	0·1
Total	72·3

The difference (27·7 %) should be lignin, which agrees with the usual figure of 28–30 % found for spruce by direct estimation with hydrochloric acid. It is obvious, therefore, that Mehta has isolated only a fraction of the lignin present in the wood.

Part of the deficiency in total lignin-content can be accounted for by the retention of a portion of the lignin in the cellulose residue after heating with caustic soda, and the remainder is to be found in the water-soluble body present in the acid filtrate after precipitation of the lignin, which may be a decomposition product of lignin. The hemicellulose portion (3·8 %) was found to be separable into two parts, a part precipitated by acid, and a water-soluble fraction, precipitated by the addition of 95 % alcohol. These results are in agreement with those of O'Dwyer [1926], who found that there were two hemicelluloses present in beech-wood.

SUMMARY.

1. By the action of 4% caustic soda at 10 atmospheres pressure for 1 hour on purified wood, a new type of lignin has been obtained and characterised, for which the name *metalignin* is proposed.

2. This lignin has the formula $C_{20}H_{20}O_6$ with molecular weight 356. It melts at 186° and is soluble in a range of organic solvents, differing in these respects from lignins previously described.

3. The preparation of derivatives indicates the presence of one hydroxyl, two methoxyl and two carbonyl groups. Two double linkages are apparently present.

4. Metalignin agrees in composition with the α-lignin of Klason [1920] existing as a sulphonic acid in bisulphite liquors.

5. The technique described in (1) above does not, as has been claimed [Mehta, 1925], give a quantitative separation of lignin.

One of us (E. C. B.-W.) desires to thank the Trustees of the Dixon Fund of London University for a grant towards the expenses of this investigation.

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XLIII. STUDIES ON CALCIUM METABOLISM.

I. THE ACTION OF THE PARATHYROID HORMONE ON THE CALCIUM CONTENT OF THE SERUM AND ON THE ABSORPTION AND EXCRETION OF CALCIUM.

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(*Received February 1st, 1927.*)

The numerous references to the low calcium content of the serum in *tetania parathyreopriva*, and more particularly the recent work of Collip [1925] on the extraction and action of the parathyroid hormone, make it clear that one action, at any rate, of the parathyroid glands is to control the concentration of calcium in the blood. So much may be taken as definitely established, but of the mode in which this control is exercised little is known. Clearly the parathyroids may affect the absorption or excretion of calcium, or may exert some controlling action on the equilibria between the different forms of combination in which calcium exists in the blood and between the concentrations in the blood and in the tissues.

In view of the ease with which the low serum calcium of *tetania parathyreopriva* can be raised by administration of calcium salts, it appears unlikely that the parathyroids act by controlling calcium absorption. Some doubt exists, too, as to whether they have any effect on the excretion of calcium. Thus Kishi [1924] found that after parathyroidectomy there was increased urinary, but unchanged faecal, excretion of calcium; MacCallum and Voegtlin [1909] found an increased faecal excretion, and Greenwald and Gross [1925, 1] found the total excretion to be decreased. These last authors, too [1926], state that long-continued administration of parathyroid to normal dogs brings about an increased calcium excretion.

The third possibility is one accepted by Greenwald and Gross [1925, 2], who have suggested that the parathyroid hormone is, or is necessary for the preparation of, the substance which keeps in solution the large excess of $Ca_3(PO_4)_2$ which Holt, La Mer and Chown [1925] have shown to be present in the blood. The excess of calcium excreted during long-continued administra-

tion of parathyroid they believe to be derived, ultimately, at any rate, from the bones.

In the work recorded in this paper we have aimed at obtaining some direct evidence as to the mode in which the parathyroid hormone exerts its action. The method of measuring the total excretion over a long period, first without and later with administration of parathyroid, is open to the objection that the differences usually observed are so slight as to be not far removed from the experimental error. They are not, moreover, easy of interpretation, for the faecal excretion is composed both of unabsorbed residues and of calcium re-excreted into the gut.

The effect of haemorrhage on the serum calcium.

Since many of our projected experiments necessitated the frequent drawing of blood samples and consequently involved considerable haemorrhage, it seemed advisable to test first the effect of haemorrhage itself on the calcium content of the serum.

Clark [1920], working with rabbits, found that extensive haemorrhage was followed by a considerable fall in the serum calcium, amounting to as much as 12 %.

We intended to use cats in our own experiments and therefore repeated Clark's work with these animals. To our surprise we found that, although the animal was bled freely at intervals of half an hour, there was no reduction in the serum calcium even at death. Numerous repetitions gave identical results; even with a loss of 60 % of the estimated total blood volume and a correspondingly large loss of haemoglobin, the serum calcium was never decreased by an amount greater than the experimental error of the method employed (that of Kramer and Tisdall [1921]). The results of these experiments are given in Table I.

The uniformity with which this effect was obtained determined us to repeat the experiments with the same species as was employed by Clark. In every case the results entirely confirmed Clark's observations.

There appears, therefore, to exist a definite species difference. The experimental conditions are identical in each series, the same anaesthetic being used, blood being taken at similar intervals and in similar amounts (with respect to the total blood volume), and the calcium estimations being carried out by the same method. We can offer no explanation of the difference, but it would seem to be in some obscure way connected with the susceptibility of the species to the action of the parathyroid hormone, for Greenwald and Gross [1926] have reported that, while cats, rabbits and rats are all more resistant than are dogs, rabbits and rats are even more resistant than are cats.

Table I. *The effect of haemorrhage on the serum calcium in the cat.*

No.	Weight of cat g.	Time min.	% haemoglobin *	Corpuscle volume % (haematocrit)	Mg. Ca per 100 cc. serum	Total haemorrhage cc.	% reduction in serum Ca	% reduction in haemoglobin
I	2800	0	66	35	9·90	—	—	—
		30	55	32	9·84	—	—	—
		60	53	29	9·87	—	—	—
		90	50	24	9·90	47	Nil	15
II	3000	0	65	31	10·3	—	—	—
		40	—	—	10·25	—	—	—
		80	—	—	10·3	—	—	—
		120	50	25	10·3	—	—	—
		150	—	—	10·4	—	—	—
		180	40	21	10·3	81	Nil	37·4
III	3000	0	70	—	9·5	—	—	—
		60	—	—	9·5	—	—	—
		120	—	—	9·5	—	—	—
		180	47	—	9·5	57	Nil	33
IV	2980	0	66	44	9·90	—	—	—
		40	—	—	9·90	—	—	—
		80	—	—	9·90	—	—	—
		120	—	—	9·90	—	—	—
		150	50	30	9·90	80	Nil	24
V	3000	0	67	—	11·0	—	—	—
		60	—	—	10·95	—	—	—
		120	—	—	10·95	—	—	—
		180	40	—	11·0	90	Nil	40
VI	3020	0	—	—	9·25	—	—	—
		60	—	—	9·25	—	—	—
		120	—	—	9·25	—	—	—
		180	—	—	9·25	89	Nil	—

Table II. *The effect of haemorrhage on the serum calcium in rabbits.*

No.	Weight of rabbit g.	Time min.	% haemoglobin *	Corpuscle volume % (haematocrit)	Mg. Ca per 100 cc. serum	Total haemorrhage cc.	% reduction in serum Ca	% reduction in haemoglobin
I	1500	0	62	30	12·90	—	—	—
		60	36	23	10·40	—	20	42
II	1000	0	53	32	11·20	—	—	—
		60	44	25	10·40	—	—	—
		100	38	23	9·50	—	14·4	28·5
III	2000	0	60	—	13·7	—	—	—
		30	52	—	13·2	—	—	—
		60	48	—	12·5	—	—	—
		90	40	—	12·4	44	8·6	33·3
IV	1950	0	60	27	11·5	—	—	—
		60	50	—	11·0	—	—	—
		120	20	15	10·0	33	10	66
V	1750	0	50	27	16·4	—	—	—
		75	40	21	13·6	30	17	20
VI	1690	0	60	28	11·9	—	—	—
		30	—	—	10·9	—	—	—
		90	—	—	10·5	—	—	—
		150	44	19	10·3	48	13·4	26·6

* Haemoglobin estimated colorimetrically against human standard.

The effect of parathyroid hormone on the blood serum of cats.

The parathyroid hormone used in our earlier experiments was kindly presented to us by Dr McNee, who had obtained it from Dr Collip; later we employed the commercial preparation "Parathormone Lilly," extracted by Collip's method. Table III shows the effect of subcutaneous injections of these preparations on cats.

Table III. *Effect of Collip's parathyroid extract, given subcutaneously, on the serum calcium in cats.*

(0·5 cc. (10 units) parathyroid extract given after withdrawal of first blood sample.)

Exp.	Weight g.	Mg. Ca per 100 cc. serum		
		Before injection	15 hr.	Increase
I	2110	10·2	12·4	2·2
II	2440	9·7	11·9	2·2
III	2800	9·8	12·8	3·0
IV	3360	11·0	14·2	3·2
V	2800	10·4	14·1	3·7

10 units extract = amount necessary to produce in 15 hours a rise of 5 mg. per 100 cc. in a 2 kg. dog; therefore the extract is approximately half as active in cats as in dogs.

In every case it will be noted the serum calcium rose to an extent which, though less than would, presumably, have been observed in dogs, was well beyond the limits of experimental error and was undoubtedly real. The effect of intravenous injection of the hormone was next investigated. The cat was anaesthetised by means of paraldehyde and ether and a sample of blood having been withdrawn from the carotid, parathyroid hormone was given intravenously and thereafter blood was drawn at frequent intervals.

Table IV.

A. *Effect of intravenous injection of "Parathormone" in cats anaesthetised with paraldehyde and ether.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected cc.	Mg. Ca per 100 cc. serum		
				30 min.	120 min.	160 min.
I	2210	10·1	0·5	10·9	11·8	11·1
II	2900	10·0	0·5	11·6	12·0	10·4
III	3300	10·4	1·0	10·9	12·0	11·6

B. *Effect of intravenous injection of "Parathormone" in decerebrate and pithed cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected cc.	Mg. Ca per 100 cc. serum	
				45 min.	90 min.
I	3400	10·30	0·6	10·70	11·65
II	2890	9·6	0·6	11·70	12·00

The results of these experiments (Table IV) show that under these conditions the hormone exerts its action much more rapidly than when given hypodermically, the maximum rise in the serum calcium being attained in about 2 hours. The magnitude of the rise, however, is not greatly increased.

In Table IV (B) we also give the results of experiments in which parathyroid hormone was given intravenously to cats after decerebration which included removal of the pituitary gland, the spinal cord having also been destroyed. The rise in the serum calcium was of the same magnitude as that in anaesthetised animals (anaesthesia, as is shown *inter alia* by the results of the haemorrhage experiments, is without appreciable effect on the serum calcium). It is evident, therefore, that the effect of the parathyroid hormone is not exerted through the central nervous system.

We may remark at this point that *in vitro* we have observed a rapid inactivation of the hormone following its exposure to air (Table V).

Table V. *Effect of subcutaneous injection of Collip's parathyroid extract, which had been exposed to the air and kept at 37° for 24 hours, on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	Parathyroid extract injected (cc.)	Mg. Ca per 100 cc. serum 15 hr.
I	1980	9·5	0·5	9·5
II	2350	10·5	0·5	10·5

It is perhaps significant that the intravenous injection of sodium bicarbonate produces an effect exactly opposite to that of parathyroid. There is a rapid fall in the serum calcium reaching its maximum in some two hours, and thereafter returning to normal. Further, the two effects can be made to counterbalance each other exactly. As is shown in Table VI the simultaneous injection of sodium bicarbonate and of "Parathormone" (shown by control

Table VI.

A. *The effect of intravenous injection of sodium bicarbonate on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	$NaHCO_3$ injected g.	Mg. Ca per 100 cc. serum 30 min.	75 min.	100 min.
I	2110	10·8	0·8	10·0	9·4	8·8
II	2750	8·9	1·0	7·4	7·4	7·5

B. *The effect of simultaneous intravenous injection of $NaHCO_3$ and "Parathormone" on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" (cc.) and $NaHCO_3$ (g.) injected	Mg. Ca per 100 cc. serum 30 min.	75 min.	100 min.
I	2750	8·8	0·7 cc. 1·0 g.	8·75	8·85	8·8
II	1870	10·2	0·7 cc. 1·0 g.	10·0	10·2	10·0

experiments to be active) leaves the serum calcium unaltered. Stewart and Haldane [1924] had previously found that oral administration of sodium bicarbonate caused a lowering of the human serum calcium (a result which we have confirmed), whereas oral administration of calcium chloride or of ammonium chloride, both of which produce an acidosis, caused a rise in the serum calcium.

These facts inevitably suggested the possibility that the action of "Parathormone" might be in some way connected with the neutrality-regulating mechanism. This idea, however, appears to have been partly disposed of by the recent work of Cantarow, Caven and Gordon [1926], who find that "Parathormone" is without effect on the CO_2-combining power of the blood.

Incidentally we may remark that oral administration of sodium bicarbonate affords two means of effecting a lowering of the blood calcium. By producing a decreased acidity of the intestinal contents it militates against the absorption of calcium; by bringing about an increased absorption of sodium, and hence an alkalosis, it may cause a direct lowering of the blood calcium. Following intravenous injection of sodium bicarbonate, the fall in the blood calcium can only be due to the alkalosis.

The effect of "Parathormone" on the absorption of calcium.

The direct measurement of the absorption of calcium is beset with many difficulties. Not only is the faecal output a combination of unabsorbed residues and of re-excreted calcium, the relative amounts of each being unknown, but the amount retained—which is all that can be measured—is obtained by difference from estimations of the total intake and total output, and is never more than a small fraction of either. The margin of error in such experiments is therefore considerable. Nor is it desirable to take the urinary excretion as an index of the amount of calcium absorption since it varies very considerably from day to day even under apparently standard conditions. The method of Bergeim [1926] undoubtedly gives a possible means of estimating absorption, but was published only after our experiments were under way. Indeed, excellent though it appears to be for many purposes, we believe it to be less satisfactory for our particular problem than the method we have employed. Briefly, our method consists in the complete removal of the alimentary canal distal to the oesophagus, *i.e.* the whole of that portion from which absorption may conceivably take place. If, under these conditions, the parathyroid hormone is able to exert its full effect on the serum calcium, it must mobilise calcium from some internal source or, possibly, act by controlling the rate of excretion. On the other hand, its inability to raise the serum calcium would indicate that normally the hormone draws on an external supply.

The technique of the experiments was as follows. Paraldehyde and ether were used to produce anaesthesia. The abdomen having been opened, the duodeno-jejunal junction was identified and the gut severed between two

ligatures at this point. The rectum was then drawn up out of the pelvis and cut across between ligatures close to the anus. The superior and inferior mesenteric arteries were ligated and the small and large intestine removed entirely. The duodenum was separated from the head of the pancreas, branches of the pancreatico-duodenal arteries being ligated. A series of ligatures were tied along the lesser and greater curvatures of the stomach from the pylorus to the cardia occluding the gastric vessels in the lesser omentum, and in the anterior layer of the greater omentum. The lower end of the oesophagus was ligated and cut across. The pylorus and first part of the duodenum were carefully separated from the portal vein, superior pancreatico-duodenal, splenic, and hepatic arteries, and the stomach and duodenum were removed by severing the omenta along the curvatures of the stomach. In this way the alimentary canal distal to the oesophagus was dispensed with, leaving the liver, spleen, and pancreas *in situ*, receiving an almost intact blood supply, that portion only of the portal circulation arising in the gut having been interfered with. The integrity of the arterial supply and venous return to these organs was in every case proved by the presence of arterial pulsation and by inspection of the venous flow. Where exclusion of liver, spleen and pancreas from the circulation was desired, the procedure was similar to that employed for evisceration alone, but it was unnecessary to separate the duodenum from the head of the pancreas. The hepatic artery and portal vein were ligated and cut in the free border of the lesser omentum, and the spleen and pancreas were removed entirely, after ligation of their respective arteries and veins.

At intervals blood was withdrawn through a cannula in the carotid artery, and injections were made through a cannula inserted in the external jugular vein. The animals were kept on artificial respiration and, despite the extensive surgical interference, their condition remained satisfactory throughout the experimental period. It was necessary, as a preliminary to the actual experiments, to ensure that in them the only variable condition was the presence or absence of added parathyroid hormone, since there were two other factors which might conceivably have caused an alteration in the calcium content of the blood. Firstly, it had to be shown that the operative shock was without effect in this direction. Secondly, in removing the whole of the alimentary canal, we had not only prevented absorption of calcium, but had also cut off one of the possible excretory routes. Control experiments in which evisceration was performed but no parathyroid hormone given showed that, over a period of three hours, the serum calcium remained absolutely unchanged. Whether the two factors are individually without effect, or whether their effects cancel one another was not determined, but was, for our purpose, a matter of no importance.

The results of a number of experiments on the effect of parathyroid hormone on the serum calcium of eviscerated cats are given in Table VII.

It will be noted that in every case the hormone produced a rise in the serum calcium, and comparison of these results with those given in Table IV shows

that the magnitude of the rise is as great in eviscerated as in normal animals. It seems fair, then, to conclude that the parathyroid hormone exerts its influence on the calcium content of the blood without drawing on external sources of calcium, *i.e.* without stimulating calcium absorption. Further, the liver, spleen and pancreas, have no special function as internal sources of calcium (though of course they may be used as reserves in the same way as other tissues) nor do they appear to influence the action of the hormone in any way.

Table VII.

A. *The effect of intravenous injection of "Parathormone" on the serum calcium of eviscerated cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected (cc.)	Mg. Ca per 100 cc. serum 45 min.	Mg. Ca per 100 cc. serum 90 min.
I	2760	9·0	0·7	9·8	11·2
II	2600	9·4	,,	11·2	11·8
III	3500	8·4	,,	11·4	10·2
IV	2990	9·6	,,	10·6	10·8
V	3330	10·0	,,	11·6	11·2
VI	3290	8·9	,,	10·0	10·8

B. *The effect of evisceration and the removal of the liver, spleen and pancreas on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before removal		Mg. Ca per 100 cc. serum 45 min.	Mg. Ca per 100 cc. serum 90 min.
I	2860	9·80	Intestine, liver,	9·80	9·80
II	3950	10·50	spleen and pan-	10·45	10·45
III	3800	9·90	creas removed	9·85	9·90

C. *The effect of "Parathormone" on the serum calcium of cats in which the liver, spleen, pancreas, intestine and stomach have been removed.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected (cc.)	Mg. Ca per 100 cc. serum 45 min.	Mg. Ca per 100 cc. serum 90 min.
I	4050	9·80	0·6	11·00	11·35
II	2980	10·30	0·6	10·70	11·65
III	4010	9·90	0·6	10·60	11·10

D. *The effect of intravenous injection of sodium bicarbonate on the serum calcium in cats in which the liver, spleen, pancreas, stomach and intestine have been removed.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	$NaHCO_3$ injected g.	Mg. Ca per 100 cc. serum 45 min.	Mg. Ca per 100 cc. serum 90 min.
I	3950	10·50	1	8·80	8·80
II	3100	9·50	1	8·30	7·90

The effect of the parathyroid hormone on the excretion of calcium.

If the parathyroid hormone acts by controlling the rate of calcium excretion, then it can only produce an increase in the serum calcium by diminishing the excretion; any increase in the excretion can only be a secondary effect due to the increased calcium concentration in the blood, the excess calcium being derived from the tissues (since increased absorption has been ruled out).

Calcium is undoubtedly excreted by the kidneys, but we desired to assure ourselves definitely that, as stated in the literature, it is also excreted through the epithelium of the large intestine. Further, if such intestinal excretion took place, we needed quantitative data. As has previously been stated, the calcium content of the large intestine represents both the unabsorbed residue and any excreted calcium; to estimate the latter directly it is necessary to exclude the former. To secure this end we adopted the following method.

The cat was anaesthetised by paraldehyde and ether, and placed on artificial respiration throughout the experimental period. Cannulae were inserted into the carotid artery and the external jugular vein. The abdomen having been opened, the small intestine was cut across between two ligatures close to the ileo-caecal valve. The large intestine was brought out and the pelvic colon cut across at its junction with the rectum, all bleeding points being ligated. A large bore cannula was inserted into the lip of the caecum. The isolated loop of large intestine was thoroughly washed out with warm distilled water through the cannula, the removal of solid and semi-solid material and mucous being aided by gently squeezing the bowel with the fingers. Washing was continued until a clear sample was obtained, and this was reserved for analysis. After every washing a final clear sample was collected separately and analysed. Only those experiments were considered in which this sample contained merely traces of calcium and so indicated that the washing had been complete.

Table VIII. *The excretion of calcium by the large intestine and by the kidney in the cat.*

Exp.	Weight of cat g.		Mg. Ca in intestinal washings	Mg. Ca in urine	Mg. Ca per 100 cc. serum: At start of exp.	10 min. after injection	At close of exp.
I	2900	1st 3 hours	0·68	0·21	—	—	—
		2nd 3 hours	0·66	0·14	—	—	—
II	2540	1st 3 hours	0·88	0·17	—	—	—
		2nd 3 hours	0·90	0·19	—	—	—
III	3010	1st 3 hours	0·33	0·22	—	—	—
		5 cc. 10 % $CaCl_2$ injected intravenously ...	—	—	10·2	22·0	15·0
		2nd 3 hours	4·27	0·25	—	—	—
IV	2700	1st 3 hours	0·68	0·16	—	—	—
		5 cc. 10 % $CaCl_2$ injected intravenously ...	—	—	—	—	—
		2nd 3 hours	14·0	0·15	—	—	—
V	2890	1st 3 hours	0·30	0·21	—	—	—
		5 cc. 10 % $CaCl_2$ injected intravenously ...	—	—	9·0	21·0	10·0
		2nd 3 hours	9·60	0·23	—	—	—

After this preliminary washing the isolated loop of intestine was replaced within the abdomen, with the two ends slightly protruding, so that any contents would not escape. The washing was repeated after three and after six hours. In a number of experiments a cannula was inserted into the bladder and samples of urine collected (with washing) at the same time as the intestine was irrigated. Table VIII, giving the results of experiments of this type, shows

that calcium was excreted into the gut and that this excretion occurred at a fairly constant rate. Further, it was usually greater than the urinary excretion for the same period.

It remained possible that the calcium found in the large intestine did not constitute a true excretion, but had merely been secreted along with the mucus. To test this point, we allowed a preliminary control period of three hours, then injected calcium salts intravenously, and again washed out the gut after a further three hours. After the injection of calcium salt the excretion of calcium into the intestine was enormously increased although there was no corresponding increase in the mucous secretion. The urine did not show any comparable increase in calcium content (Table VIII). It seems, then, that not only is calcium excreted by way of the large intestine, but that this is the main excretory route.

The effect of the parathyroid hormone was tested by a method exactly similar to that described above, an injection of the hormone being given at the end of a three-hour control period. That the hormone was active was shown in each case by the withdrawal of blood samples at intervals and estimation of the serum calcium. The results, set forth in Table IX, showed the hormone to have little or no effect on the intestinal excretion of calcium.

Table IX. *The effect of intravenous injection of "Parathormone" on the excretion of calcium by the large intestine in cats.*

Exp.	Weight of cat g.	Mg. Ca in large intestine after 1st 3 hr. period	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected cc.	Mg. Ca per 100 cc. serum 60 min. after injection	Mg. Ca in large intestine after 2nd 3 hr. period	Mg. Ca per 100 cc. serum 3 hr. after injection
I	2790	0·74	10·6	2	11·4	2·0	12·0
II	2400	0·70	9·9	2	11·1	0·72	11·5
III	2300	0·64	9·8	2	11·0	0·80	11·7
IV	3050	0·60	10·0	2	11·2	0·60	11·8

In only one experiment was a definite increase obtained, but, on the other hand, in no case was there a decrease. The latter, as has been pointed out, is the important finding, and indicates clearly that the parathyroid hormone does not control the rate of excretion of calcium by way of the large intestine. The fact that little or no increase in the calcium excretion was observed in these experiments is not surprising in view of the small rise in the serum calcium produced by the parathyroid hormone compared with that following the injection of calcium salts.

Indeed when one considers the amount of calcium excreted during the control period, it seems hardly possible that the parathyroid hormone could produce the observed rise in the serum calcium by diminishing this amount. The average of a number of experiments shows that the total excretion of calcium in the control period of three hours is 0·8 mg., the greatest excretion observed being just over 1·0 mg. Now in all our experiments the serum calcium

has been at least 1·0 mg. per 100 cc. higher at the end of the three-hour period than at the beginning, and the net rise has usually been greater than this. The smallest cat used in these experiments weighed 2500 g., which means an approximate blood volume of 200 cc. and therefore at least 100 cc. of serum. Hence an increase of 1 mg. per 100 cc. in the serum calcium involves the mobilisation of 1·0 mg. calcium at least, an amount greater than the total excretion during the period in which this rise has taken place. Obviously then, control of the rate of excretion cannot be the main mode of action of the parathyroid hormone. When one adds the experimental finding that the intestinal excretion, which accounts for much more than half of the total, is not diminished at all, it follows that the hormone does not act by controlling the rate of calcium excretion.

Discussion.

It is evident that the increase in the serum calcium following the administration of parathyroid extract is due to a withdrawal of calcium from the body tissues, for we have shown that the increase takes place without any diminution in the excretion, and without the possibility of any absorption from the alimentary canal.

Of the total serum calcium only about 60 % is readily diffusible through a collodion membrane [Cushny, 1920]. This readily diffusible fraction is apparently identical with Vines' "active calcium" [1924] which is precipitated by one equivalent of ammonium oxalate. Measurements of the ionic calcium [Neuhausen and Marshall, 1922] show that only 10–20 % of the total exists as ions. It is reasonable to suppose that the calcium mobilised by the parathyroid is readily diffusible, and that, therefore, the administration of parathyroid will be followed by a rise in the ratio of diffusible to total calcium in the serum. That such is actually the case has been stated by Vines [1924], and we have confirmed his statement [Stewart and Percival, 1927]. Several workers have shown that parathyroidectomy produces a relatively greater fall in the diffusible than in the non-diffusible calcium [Salvesen and Linder, 1924; Trendelenburg and Goebel, 1921; Moritz, 1925]. Hence it seems that on the amount of parathyroid hormone depends primarily the concentration of the readily diffusible calcium. This conclusion is to some extent in agreement with the suggestion of Greenwald and Gross [1925, 2] that the parathyroid hormone is, or is necessary for the production of, a substance (which Greenwald [1926] considers to resemble citric acid) capable of retaining in solution the excess $Ca_3(PO_4)_2$ which Holt, La Mer and Chown [1925] have shown to be present in blood.

The calcium drawn into the blood when parathyroid is administered must come from the soft tissues or from the bones—or from both. Using whole animals, it seems impossible to gain any direct information as to which alternative is taken, except by long-continued administration of the hormone, with, perhaps, actual tissue analysis. In short experiments the quantities

involved are hopelessly small. Even in long ones the large number of analyses of intake and output, with the relatively small differences on which to found conclusions as to the quantity of calcium retained or lost, render the results of doubtful value. Greenwald [1926], however, in an experiment of this kind claims that the amount of calcium lost during the period in which parathyroid was administered could only have come from the bones. He points out, however, that the blood may have drawn its extra calcium from the soft tissues which thus made up their supply at the expense of the bones. The rapidity with which parathyroid produces a rise in the blood calcium suggests, indeed, that the soft tissues form the primary source of supply.

We are at present engaged in a series of experiments by means of which we hope to gain some further information as to the immediate source of the calcium mobilised by the parathyroid hormone.

SUMMARY.

1. Extensive haemorrhage causes a lowering of the serum calcium in rabbits but not in cats. Rabbits are less susceptible to the action of parathyroid extract than are cats.

2. Parathyroid extract ("Parathormone") raised the serum calcium of cats when injected subcutaneously, and more rapidly when injected intravenously. The action is prevented by the simultaneous injection of sodium bicarbonate.

3. Since the parathyroid hormone can exert its full action on the serum calcium even after complete removal of the alimentary canal, it is concluded that the action does not consist in controlling the rate of calcium absorption.

4. The large intestine provides the main excretory route for calcium.

5. Following administration of parathyroid, and while the serum calcium is high, there is no diminution in the excretion of calcium. The parathyroids, therefore, do not act by controlling the rate of excretion.

6. The liver, spleen, pancreas, pituitary, thyroid, and central nervous system appear to have no special function in connection with the effect of parathyroid on the blood calcium.

7. It is considered that the parathyroid hormone controls the distribution of calcium between the blood and the tissues by regulating the proportion of the total serum calcium which is readily diffusible.

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XLIV. ON LIVER AMYLASE.

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In view of certain peculiarities in the relation of the velocity of hydrolysis of starch by the amylase of germinated barley to its concentration [Eadie, 1926], it was decided to investigate the relations which hold in the case of liver amylase and glycogen, in the hope of throwing more light on the physiology of glycogen in the liver and the regulation of carbohydrate metabolism. Early in this work the disconcerting fact was disclosed that the optimum hydrogen ion concentration varied with the preparation used, and was now at p_H 7 and now at p_H 6. Earlier workers have uniformly stated it to be about p_H 7 (see for example, Holmbergh [1923], who gives references to the earlier papers), although careful search of the literature shows that this has not always been justified by their results. During the course of these experiments a paper by Davenport [1926] was published in which this peculiarity was noticed when liver amylase acted on starch.

Since the liver preparations used contained more or less blood, it seemed possible that there were really two or more amylases present. The amylase of the blood [Euler, 1922] is probably derived for the most part from the pancreas, and pancreatic amylase has an optimum about p_H 7. This suggested that the more alkaline optimum in the case of the liver preparations was due to amylase derived from the blood present in the liver, and that the liver amylase itself was responsible for the more acid optimum.

Methods.

The extent of hydrolysis was estimated by determining the amount of sugar produced, using the method of Shaffer and Hartmann. Reducing power was calculated as glucose. The objections raised by Visscher [1926] to the use of this method in the presence of phosphate buffers apparently do not apply when the concentration of phosphate is as low as in these experiments [Davenport, 1926].

The liver was prepared by a method similar to that described by Holmbergh [1923]; it was thoroughly ground, then treated twice with acetone, once with a mixture of equal parts of acetone and ether, and finally twice with ether. The quantities and times recommended by Holmbergh were not strictly followed. After drying overnight the powder thus obtained was extracted for several days with 50 % glycerol filtered and dialysed against running water for about five hours. The resultant solution was frequently found to be unstable, but it could always be used for 24 hours.

Clark and Lubs' phosphate buffers were used and checked electrometrically.

Liver from various sources was tried, but the most active preparations came from the rat rather than from the pig or sheep.

The glycogen used was prepared by Pflüger's method, for the most part from rabbit liver, but in a few experiments that from mussels was used. No difference was found between the glycogens from these two sources.

During hydrolysis the reacting solutions were kept in a thermostat at 37°.

Effect of perfusion on rat liver[1].

A rat liver was treated by the method just outlined after washing off the blood but without perfusion, and its activity tested in the following way.

Into each of five flasks were placed 5 cc. of 1·5 % glycogen solution, 0·5 cc. of 2·5 % sodium chloride solution, 5 cc. buffer (of varying p_H) and 15 cc. water. These were warmed to 37°, and 5 cc. of the enzyme solution were added to each. Samples removed immediately after mixing showed the absence of sugar. Samples were taken in 2, 4 and 24 hours. The results for the 4-hour samples are shown in Fig. 1 (continuous line), and those for the other two periods are in substantial agreement with these. An almost identical curve was obtained with sheep's liver. It will be noted that there are two maxima, one about p_H 6 and another about p_H 6·8 or 6·9.

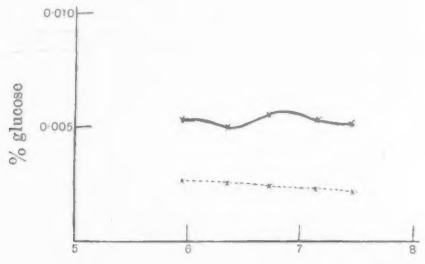

Fig. 1. Effect of perfusion.
——— Unperfused liver.
– – – – Perfused liver. The figures on the ordinate refer to the unperfused liver. For the actual values for the perfused liver see text.

Another rat was then taken and perfused for about an hour with warmed Ringer's solution. After this time the liver showed no traces of blood. Treated in an exactly similar way, it gave the results shown below.

p_H: 5·95, 6·35, 6·72, 7·14, 7·41.

Glucose % after $5\frac{1}{4}$ hours: 0·0525, 0·0510, 0·0480, 0·0465, 0·0440.

These results are plotted in Fig. 1 (dotted line) on the same scale as the other curve, but to prevent the curves overlapping, another base line is used.

[1] I am indebted to Mrs Holmes for her kindness in perfusing these livers.

The figures in the margin should each be increased by 0·0025 to apply to this curve.

Here the maximum between 6·7 and 7·1 has disappeared. Evidently the perfusion has washed out the blood and with it an amylase having its optimum about the neutral point.

Since the rat liver is rather small, I endeavoured to find a method for freeing from blood livers from the slaughter-house. For this purpose a quantity of pig's liver was finely ground, stirred up with water, centrifuged and again treated with water, the process being repeated four times.

After being treated in the usual way this preparation was allowed to act on glycogen under the same conditions as before. After 5 hours' hydrolysis the following amounts of sugar were found:

p_H: 5·95, 6·35, 6·72, 7·14, 7·41.

Glucose %: 0·0016, 0·0028, 0·0032, 0·0040, 0·0020.

This was clearly unsuccessful.

Another plan was then tried based on that used by Thunberg for washing muscle. The ground liver was placed in a tall glass cylinder covered with gauze through which a glass tube passed leading to the bottom of the cylinder. Tap water was run through the glass tube until the water coming away was clear. The residue was treated in the usual way and the extract allowed to act as before. In 4½ hours the amounts of sugar produced were:

p_H: 5·95, 6·35, 6·72, 7·14, 7·41.

Glucose %: 0·0056, 0·0050, 0·0062, 0·0056, 0·0053.

There has been a shift of the optimum towards the acid side here but not so completely as in the case of the perfused rat liver. Obviously some blood amylase is still present although in relatively less amount.

Since the rat liver was so much more active and so much easier to wash free from blood, it was decided to continue with it. Lesser [1920] considers that washing of the liver takes out some of the liver amylase, and I find it associated with considerable loss of activity. Since the pig's liver was not very active to begin with there could be very little activity left after sufficient washing to free it from blood.

It should be noted here that this change in optimum p_H is not of the kind described by Michaelis and Pechstein [1914] among others in the case of ptyalin, where it varies with the salt present in the mixture, for in all cases here the same amounts of phosphate and sodium chloride were used. Moreover, the presence of two optima in certain experiments with unwashed liver could not be explained in this way.

Influence of substrate concentration.

Fig. 2 shows the amounts of sugar formed in an hour under the following conditions. Six flasks were prepared containing 0·5 cc. of 2·5 % sodium chloride solution, 5 cc. phosphate buffer of p_H 5·95, varying quantities of

1·5 % glycogen solution and water to make a total volume of 25·5 cc. After being warmed to 37°, 5 cc. of enzyme solution were added to each.

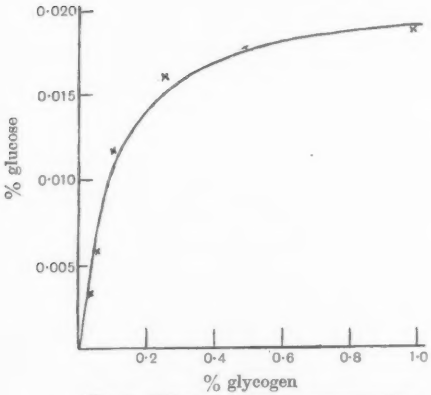

Fig. 2. Effect of substrate concentration.

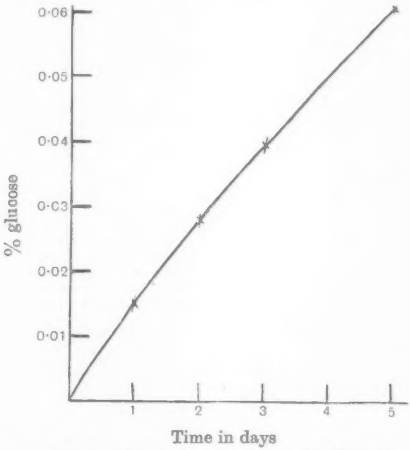

Fig. 3. Showing initial stages of hydrolysis.

That the rate of reduction is practically linear over this range is shown by Fig. 3, which refers to a solution of a similar composition containing 0·45 % of glycogen. During the time covered by the curve 0·06 % of glucose was produced, *i.e.* 10 to 15 % of the amount for total hydrolysis. This is about the extent of hydrolysis in the experiment illustrated in Fig. 2. Any deviation from the linear relation is likely to produce results which are too low in the case of the lower concentrations of glycogen and (relatively) too high in the case of the higher concentrations.

The smooth curve in Fig. 2 is based on the theory proposed by Michaelis and Menten [1913], who deduce from it the equation:

$$v = k \cdot \frac{S}{S + K_s}.$$

Here v is the velocity, S the substrate concentration, K_s the "affinity constant" or Michaelis constant, and k is another constant. The curve in Fig. 2 is plotted from this equation, putting $K_s = 0{\cdot}095$ and $k = 0{\cdot}00021$. This value for the affinity constant is of the same order of magnitude as those obtained by Michaelis in the paper quoted.

It will be seen that the agreement of the values found with those predicted from theory is within the limits of experimental error. Closer examination reveals that the first two experimental points lie below the curve and the three following above it, which might indicate a constant trend. I think that this is due to the fact that the velocity (as mentioned above) falls off somewhat with time, and affects the points corresponding to the smaller concentrations first. I have tried several other types of curve, but no other seems to fit the results nearly so well.

These results therefore differ markedly from those obtained with starch and the amylase of germinated barley, and resemble those obtained with such a system as sucrose and invertase.

An attempt was made to do this experiment with a preparation made from unwashed liver. The curve obtained resembled this one somewhat, but, as might be expected, was rather more complex and did not fit exactly any well-known type of curve.

Effect of salt concentration.

Since it is probable that most previous workers have used mixtures of blood and liver amylase to investigate this point, it was thought worth while to repeat the experiment.

Flasks were set up each containing 5 cc. of 1·5 % glycogen solution, varying quantities of 0·30 % sodium chloride solution, and water to make 20 cc. 5 cc. of enzyme solution were added to each in the manner described above. The concentration of glycogen thus was 0·25 %. The sugar formed in one hour was estimated and the results are given in Fig. 4.

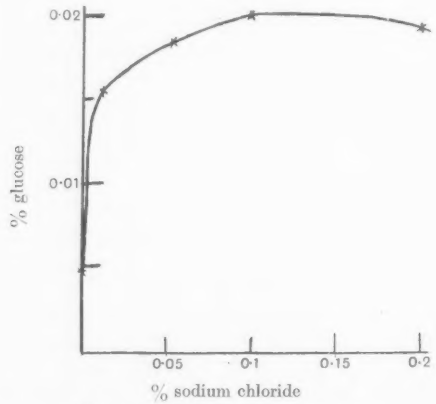

Fig. 4. Effect of salt concentration.

It will be seen that this amylase resembles closely the other amylases of animal origin in being extremely sensitive to the presence of sodium chloride. The optimum is apparently about 0·1 %.

Effect of adrenaline.

These experiments were done in the spring of 1926. Since that time two other contributions to the subject have been made. It will be recalled that Langfeldt [1921] claimed that adrenaline not only increased the activity of the liver amylase, but also altered its optimum hydrogen ion concentration.

Working with liver amylase and glycogen I was not able to find any alteration in the optimum p_H in the presence of adrenaline. There was, however, increased activity, but this disappeared when the concentration of sodium chloride in the controls and in the solutions containing adrenaline was made equal. Commercial preparations of adrenaline contain considerable amounts of sodium chloride.

Visscher [1926] using glycogen as a substrate found no alteration of the optimum p_H, but his enzyme was evidently badly contaminated with blood amylase, and he did not discover the chloride effect. Later, Davenport [1926] reported that with starch as a substrate adrenaline had no effect, apart from the sodium chloride contained in its preparations. Davenport used several enzyme preparations, some of which contained blood amylase in large quantity while others did not. I have found a similar variation in the blood amylase content in preparations made by Holmbergh's method; the method used by Davenport evidently gives similar results.

It appears therefore that Langfeldt's claims are not substantiated.

Discussion.

In view of the failure of earlier workers to recognise that their preparations of liver amylase might be badly contaminated by that from the blood, a good deal of their work requires revision.

One of the most detailed studies of the enzyme is that by Holmbergh [1923]. He states that the optimum p_H in the presence of sodium chloride and phosphate buffer is 6·9. This indicates that his preparation contained so much blood amylase that its activity overshadowed that of the liver; consequently his estimates of the quantity of amylase in the liver are probably much too high. Similar suspicion also attaches to other estimates for in no case is there proof that liver amylase alone is being estimated. Indeed, to estimate the amount of liver amylase is a matter of some difficulty; no method of washing out the blood seems satisfactory, because, as Lesser and Kerner [1920] have shown, thorough perfusion also washes out some of the true liver amylase.

My experiments have also some bearing on the question of glycogenolysis. For the problem of how amylase and glycogen can exist together in the liver cell, several solutions have been offered. One of these is that, to quote Macleod [1922], "glycogenolysis in the liver cell could be set up by the local production of a certain amount of acid. Such a liberation of free acid could be brought about by a curtailment in the arterial blood supply of the hepatic cell..." and vasoconstriction either from adrenaline or nervous stimulation is suggested as a probable cause. The argument includes the remark that "diastatic enzymes are particularly susceptible to the reaction (c_H) of their environment, a very slight degree of acidity favouring and a trace of alkalinity markedly depressing their activities." It is apparently assumed that at the normal p_H of the liver cell the enzyme is inactive, and that its activity enormously

increases with the degree of acidity. It seems unlikely that a physiological process such as vasoconstriction would lead to such a change as from p_H 7·4 to 6. Yet we see that the enzyme is by no means inactive at the former p_H, and in changing to the latter its activity increases by less than 25 % (cf. Fig. 1). A change from p_H 7·4 to 6·7, which is probably much closer to changes occurring in the living cell, increases the activity by about 10 %.

Since the rate of hydrolysis is very nearly constant over a considerable period at the beginning, the figures from which these calculations are made give a pretty close measure of the initial rates, and may be used in place of them without any error which would affect this argument in the slightest.

The liver amylase itself is either not an active enzyme compared with that of the blood, or is present in very small concentrations, for the presence of blood in the organ from which the preparation is made is able to mask it almost completely. It is very much weaker than that of the pancreas. These facts, in connection with the flatness of the activity-p_H curve, render the explanation quoted above much less probable than it appeared to be in the absence of more detailed knowledge of the enzyme.

Lesser [Lesser and Kerner, 1920; Lesser, 1920, 1921] has advocated another view, viz. that the glycogen and amylase are, as it were, "locked up" in different compartments of the cell. This explanation unfortunately adds little to our knowledge because it suggests no mechanism whereby the two are kept separate. Lesser finds a loss of activity on perfusion which he attributes to the washing-out of liver amylase only, for he maintains, on the basis of experiments by himself and by Fröhlich and Pollak, that the amylase of the blood can have no action on the liver glycogen. He reports [1921] an experiment in which he perfused livers with solutions of varying p_H and measured the hourly output of glucose. The figures for the first hour's perfusion show maximal amounts at p_H 7·44 and 5·66. The figures for the second, third and fourth hours show maxima at 6·45. (There was no solution of p_H intermediate between 5·66 and 6·45.) These values refer of course to the perfusion fluid and are not to be taken as indicating very accurately the conditions under which the actual hydrolysis occurs, although they must influence it to a great extent and one which becomes greater as time goes on.

These facts would be simply explained if we admit that the blood amylase is active during the first hour, but is washed out by perfusion much more rapidly than the liver amylase. If this be admitted, I suggest that it is quite possible that blood amylase may act on glycogen under physiological conditions.

SUMMARY.

1. The optimum c_H for liver amylase is shown to be at least as high as that indicated by p_H 6, and the previously accepted value is attributed to the amylase of the blood.

2. The effect of substrate concentration on the activity has been determined and shown to be in accordance with the theory of Michaelis.

3. The effect of sodium chloride is shown to be similar to that found with other amylases of animal origin, and the effect of adrenaline solutions is found to be due solely to the sodium chloride contained in them.

4. The bearing of these experiments on the problem of glycogenolysis in the liver is discussed.

I should like to acknowledge my indebtedness to Sir F. G. Hopkins and Mr J. B. S. Haldane for much helpful criticism.

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XLV. THE BEHAVIOUR OF WHOLE BLOOD TOWARDS MALTOSE *IN VITRO*.

By ALEXANDER HYND and MARJORIE GIFFEN MACFARLANE.

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(*Received February 23rd, 1927.*)

Maltose is the only disaccharide which, on subcutaneous injection, has been found capable of alleviating the symptoms caused by insulin. As has been shown by Herring, Irvine and Macleod [1924], its action, in this respect, in the case of mice is almost as efficient as that of glucose or mannose, but with larger animals, such as rabbits, cats or dogs, it is less effective. Even with mice, it exercises its action more slowly than glucose and this would suggest that maltose requires to undergo hydrolysis before it can counteract the action of insulin. Further, the inequality in the rates of hydrolysis of maltose might account for the difference in the results obtained in different species of animals. Support for this view seemed to be furnished by the results of investigations on the maltase content of blood-serum reported by Compton [1921]. This worker found that the maltase content of the blood was not the same in all animals and he divided mammals into two groups, according to whether they had or had not maltase in their blood. Unfortunately, Compton did not include the mouse amongst the animals he investigated, and the experiments now recorded were undertaken, initially at least, to determine whether the greater efficiency of maltose in relieving mice from the effects of an injection of insulin could be correlated with a greater maltase content of the blood in this animal than in larger animals such as the rabbit or dog.

Experimental.

Our experimental procedure was very similar to that described by Compton except that whole blood was used in place of serum, and Wood-Ost's method for the sugar determinations was employed instead of Bertrand's. One of us (A. H.) is responsible for the animal experiments necessary for the research, while the other (M. G. M.) carried out the greater part of the chemical analyses. All the apparatus used (flasks, pipettes, etc.) was sterilised, and the sugar solutions (4–6 %) were prepared with water previously saturated with toluene. Blood from the following eight species of animals, namely, mouse, rat, guinea-pig, rabbit, kitten, pig, sheep and ox, was examined. The smaller animals, mouse, rat, and guinea-pig, were chloroformed and bled from the neck: the kitten was also anaesthetised and blood taken from either the carotid artery or jugular vein; the rabbit's blood was obtained from the marginal ear vein, while blood of the pig, sheep and ox was procured directly from the

slaughter-house. In the case of mice, a number of animals, usually 5 or 6, had to be used for each experiment so as to obtain a sufficient quantity of blood. A small amount of potassium oxalate was added to prevent the blood samples from clotting. One cc. of blood and 5 cc. of sugar solution were used for each experiment, the blood, in every case, being added to the sugar solution in from 2 to 5 minutes after its removal from the animal. In our earlier experiments, the reaction mixtures were incubated at body temperature, 37°, but the majority were carried out at 50°, which approaches more closely to the optimum temperature for the action of maltase [Compton, 1915].

After 5 cc. of the maltose solution and 1 cc. of blood had been incubated at 50° for 24 hours, 43 cc. saturated sodium sulphate solution were added and the mixture brought to the boil. After cooling, 1 cc. of "dialysed iron" was added to complete the precipitation of the blood proteins. Ten cc. portions of the resulting clear filtrate, which is exactly ten times more dilute than the original maltose solution, were used to determine the sugar content. Two control experiments were always carried out simultaneously. In one, 5 cc. of the maltose solution and 1 cc. of blood were mixed and immediately heated to boiling to inactivate all enzymes present. Thereafter the mixture was incubated at 50° for 24 hours and the sugar estimated as described above. In the other, 5 cc. of an approximately equivalent glucose solution and 1 cc. of blood were incubated at 50° for 24 hours, and the glucose content then determined and compared with the theoretical amount.

The experimental results obtained are summarised in the following table, in which there is also indicated the condition of the animal used.

Table of Results.

A = initial reducing power expressed as glucose in reaction mixture.
B = reducing power expressed as glucose after 24 hr. at 50° with blood.
C = reducing power expressed as glucose after 24 hr. at 50° with "boiled" blood.
D = initial concentration of glucose in control reaction mixture.
E = concentration of glucose after 24 hr. at 50° with blood.

Animal	Temp. °	A %	B %	C %	D %	E %
1. Rat (normal)	37	·616	·629	·646	—	—
2. ,, ...	,,	·616	·635	·618	—	—
3. ,, ...	50	·612	·632	·629	·608	·584
4. ,, ...	,,	·612	·620	·616	·495	·466
5. Guinea-pig (normal)	,,	·612	·560	·616	·495	·460
6. ,, ,, ...	,,	·612	·568	·600	·495	·461
7. Mouse (high carbohydrate diet) ...	,,	·328	·352	·336	·388	·388
8. ,, (low carbohydrate diet) ...	,,	·328	·352	·352	·388	·396
9. Rabbit (normal)	,,	·593	·593	·600	·495	·455
10. ,, (after daily intravenous injection of maltose for a week) ...	,,	·593	·584	·585	·388	·379
11. Kitten (excessive carbohydrate diet)	,,	·616	·623	·616	·577	·583
12. ,, (,, protein diet) ...	,,	·616	·632	·638	·577	·585
13. ,, (,, fat diet) ...	,,	·616	·633	·637	·577	·584
14. Ox (normal)	,,	·609	·625	·610	·516	·525
15. ,, ...	,,	·609	·615	·612	·516	·536
16. Sheep (normal)	,,	·595	·552	·565	·516	·519
17. ,, ...	,,	·595	·591	·578	·516	·523
18. Pig (normal)	,,	·365	·724	·355	·445	·446
19. ,, ...	37	·365	·558	·364	·445	·428

Complete hydrolysis of the filtrates obtained in the maltose experiments with both blood and "boiled" blood was also carried out as follows. To 20 cc. of filtrate 2 cc. concentrated hydrochloric acid were added, and the mixture boiled gently under a reflux condenser for half an hour. The solution was then neutralised with the *exact* amount of sodium carbonate solution, the total volume adjusted to 50 cc., and portions used for analysis. The results of these experiments need not be reported in detail, as in every case values approximating closely to the theoretical were obtained.

Further control experiments were undertaken in which 1 cc. of an extract of commercial maltase was used in place of blood. In the first of these experiments 1 cc. of extract was added to 5 cc. of the maltose solution: in the second a trace of potassium oxalate was also added to the mixture, while the third was heated to boiling immediately on mixing the maltose solution with the enzyme extract. All three were then incubated for 24 hours at 50°, and examined exactly as described for the experiments with blood. The following results were obtained.

	Reducing power expressed as glucose		
	Initial	After 24 hr. at 50°	% of maltose hydrolysed
1. Maltose + enzyme extract	0·616 %	1·071 %	87 %
2. „ + „ + oxalate ...	„	1·045	85
3. „ + boiled enzyme extract ...	„	0·694	—

The p_H of the above three mixtures as determined by the colorimetric method was identical, namely 6·7. As this method could not be applied directly to the experiments in which blood was used, the p_H of a mixture containing 5 volumes of the maltose solution and 1 volume of a phosphate buffer of p_H 7·4 was determined and found to be 6·9.

Discussion.

From the results tabulated above, it is evident that during the incubation no appreciable loss of glucose took place in any of our experiments, as a close agreement exists in every case between the values in columns D and E. Consequently the glycolytic action of the blood was a negligible factor in these experiments. Further, the values in columns A, B and C also exhibit a close agreement in the case of all the animals used except the pig. Maltase would thus appear to be absent from the blood of these animals, or only present in amounts too small to be detected by the method employed. At the same time, it is to be noted that, under identical experimental conditions, in the case of the pig, while the value for C (0·355 %) agreed with that of A (0·365 %), B was increased to an amount (0·724 %) corresponding to almost 100 % hydrolysis of the maltose used. This result is in agreement with that of other workers as to the high maltase activity of pig's blood, and also indicates that our experimental conditions were suitable for the enzyme action to proceed.

Although the p_H of the reaction mixtures was not determined, it was assumed, in view of the control experiment with the phosphate buffer referred to above, that the p_H of each mixture would be in the neighbourhood of 6·7–6·9, and as the experiments were conducted at 50°, the conditions employed were, according to the researches of Compton [1915, 1924], the optimum both as regards temperature and reaction. Additional proof of this is afforded by the result of the experiments in which an extract of a commercial sample of maltase was used. In this case 87 % of the maltose was hydrolysed. As with the addition of a trace of potassium oxalate 85 % was hydrolysed, the oxalate used to prevent clotting in the experiments with blood would exert no inhibiting action on maltase.

Consequently, the negative results obtained in the case of the mouse, rat, guinea-pig, rabbit, kitten, ox and sheep indicate that maltase is lacking from the blood of these animals. This is not altogether in agreement with the observations of other workers. Thus, while the rabbit, guinea-pig and kitten fall in his "non-maltase" group, the rat and sheep belong to Compton's "maltase" group, but Doxiades [1911], on the other hand, using a polarimetric method, gives figures indicating that rabbit's serum has a maltase activity as great as that of the sheep or dog. Bial [1893], also, at an earlier date, claimed to have demonstrated the presence of maltase in human blood by the isolation of glucose phenylosazone, although it is to be noted that no change in the polarimetric or reducing value of the solutions was detected. The discrepancy between the results of other workers and those now recorded does not appear to be due to the substitution of whole blood for blood-serum, for an experiment with pig's serum gave a much lower percentage of maltose hydrolysed (50 %) than a similar experiment with whole blood (100 %). It seems likely that the discordant results depend partly on different methods being adopted to detect the enzyme action and partly on a larger proportion of serum being employed by earlier workers. Taking the results as a whole, they do not support Compton's classification.

In our experiments we failed to detect the presence of maltase in the blood of any of the animals employed except the pig: and we did not succeed in our attempts to affect the content of blood maltase by different diets in the case of mice and kittens or by a daily intravenous injection of maltose in the case of a rabbit. Our results point to there being no distinct difference in the activity of maltase in the blood of the mouse, as compared with that of the rabbit. Consequently it follows that if maltose counteracts the effects of insulin only after hydrolysis, such hydrolysis does not occur in the blood. As it is known that an insulinised mouse responds more readily to an injection of maltose than does an insulinised rabbit, it is possible that hydrolysis is effected by an enzyme present in the tissues.

On the other hand, it may be noted that maltose, being a glucose α-glucoside, differs from other sugars (save glucose) so far employed to alleviate the symptoms caused by insulin, in having in its molecule, at one and the same

time, a *reducing group* and a *complete glucose configuration*. As the combined effect of these two factors may account for the efficiency of a compound sugar in counteracting the effect of insulin, experiments with cellobiose and gentiobiose in this connection would be of great interest. However, until further work can be carried out, it must remain an open question as to whether the efficiency of maltose in alleviating the symptoms resulting from insulin administration depends on the constitution of the compound or on its hydrolysis to glucose. In the latter case, the splitting up of the disaccharide is not accomplished by the blood maltase, but probably takes place in the tissue cells.

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XLVI. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

I. AMMONIA AND UREA PRODUCTION BY KIDNEY.

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The present work is an attempt to approach the study of the metabolism of some growing mammalian tissues. While much work has been carried out on the adult surviving or functioning organ by such methods as perfusion experiments, extirpation of an organ, etc., we know practically nothing of any chemical changes due actually to growth of any particular embryonic organ in the mammal. Probably the only way to study such changes is by means of the technique of tissue culture, where growth takes place *in vitro*. It is through this technique, suitably adapted to our needs, that we have endeavoured to attack the problem. In this communication the changes in ammonia and urea content of the embryo rat kidney are discussed, together with a note on the brain.

Technique.

A technique had to be devised which would give us sufficient material for analytical treatment; it was also necessary to use a liquid culture medium. The routine finally adopted is as follows.

A pregnant doe is anaesthetised and the two horns of the uterus removed under aseptic conditions and placed in two sterile Petri dishes. As much of the work as possible is now done inside a box with a glass top and side doors, similar to that described by Strangeways [1924]. Absolute sterility must be maintained throughout the lengthy processes involved. The embryos are removed from the uterus, decapitated and eviscerated, and, if they are well developed, it is advisable to remove the backbone. The kidneys are removed and placed in a separate dish. The embryonic tissue is chopped as finely as possible, and mixed with Ringer's solution. It is probable that different tissues require Ringer solutions of different composition, but for kidney we have used that described by Pannett and Compton [1924]. The solution is very lightly centrifuged in a hand machine. The upper layer of fluid, which will contain most of the blood cells, is removed, and the minced tissue again mixed with Ringer's solution. It may be necessary again to remove the supernatant liquid layer, but usually hard centrifuging will now leave a faintly opalescent fluid, free from cells. The removal of the blood-cells is most important, as their presence in the culture medium inhibits growth. The cell-free extract thus

obtained nearly always requires further dilution with Ringer's solution, the final strength most suitable for growth being a matter which can only be determined as the result of some experience. A little practice soon enables one to prepare extracts which are remarkably constant in their ammonia content, as the following figures (expressed in mg. ammonia-N per 2 cc. extract) will show:

0·026 0·023 0·026 0·025 0·030 0·022

The kidneys must be freed from all adhering blood, and the number required for each experiment of a series placed in separate dishes, where they are then cut into small pieces. For each experiment we have used 2 cc. of extract, and generally two kidneys. We have taken as much care as possible to use the same amount of tissue throughout any one series of experiments, but owing to the strict aseptic conditions which have to be maintained, it has been found quite impossible to use weighed amounts of tissue. The wet weight of two embryo kidneys from each litter can be determined (*e.g.* 29·5, 44·0, 25·0, 34·0 mg.), and this weight taken as representative of the weight of the remaining kidneys of that litter.

In order that growth may take place, it is necessary for the tissues to have a surface to which they can attach themselves, and for them to be kept moistened and surrounded by the culture medium without being submerged in it. It is thus necessary to use a flask or dish of such a size that the right depth of fluid is obtained when a given quantity of the medium (in this case 2 cc.) is placed in it. In a few of our early experiments we used 100 cc. conical hard-glass flasks with a layer of filter paper on the bottom, but satisfactory growth was not obtained on this, and it was therefore discontinued. Kidney tissue undoubtedly grows best on a glass surface, but if the dish is not absolutely level on the bottom there is danger that a certain amount of tissue will float in the medium, and thus be prevented from growing. We found it almost impossible to obtain dishes or flasks sufficiently even, and on the whole we have found that a very thin layer of cotton-wool has proved quite satisfactory. The pieces of tissue rest on the strands, kept moist by the medium, but in contact with the air. Rubber stoppers or dish covers must be well coated with paraffin wax to prevent evaporation and infection. Incubation at 37° is allowed to proceed for 48 hours. It is generally possible to tell by the naked eye whether or not the culture is sterile after incubation, but as a routine we make smear preparations, which are then stained by Gram's method, and examined with an oil immersion lens. If there should still be any doubt, a drop of the culture medium should be plated out on agar. No analyses have ever been carried out on any but completely sterile cultures.

The culture is filtered into a dry, weighed, hard-glass test-tube, the flask or dish washed with successive small quantities of ammonia-free water, the washings being passed through the filter and added to the filtrate. The weight of the contents of the tube is determined, and aliquot parts are taken for estimation. The filtrate should be kept in the ice-chest till required.

All apparatus for this work must be sterilised, having first been specially cleaned and prepared. In particular, it is important that no trace of alkali should be present on any of the glassware. As a matter of routine we have washed all the glass in sulphuric acid and dichromate, followed by a thorough rinsing in running water, then in distilled water, and finally in ammonia-free water. Each dish, pipette, and cork is separately wrapped in tissue-paper before being sterilised.

For estimation of the ammonia we have used Stanford's method [1923] which we have found to be very accurate until amounts of ammonia-N less than 0·012 mg. are being measured when it is liable to a 10 % error. For urea we have incubated an aliquot part of the filtrate with urease paper [Folin, 1923], and have then estimated the total urea- and ammonia-N, the urea-N being obtained by difference.

Experimental.

An elaborate system of controls is necessary:

A. 2 cc. of medium, without incubation.

B. 2 cc. of medium, plus the same amount of tissue as that used for the growth flasks (*i.e.* generally two kidneys), without incubation.

C. 2 cc. of medium, incubated for 48 hours.

A and B are kept at 0° until required.

Thus the total control will be given by:

$$A + (B - A) + (C - A) = B + (C - A),$$

where B will give the amounts of urea and ammonia nitrogen in the medium and tissue before incubation, and (C − A) will give any due to autolysis of the medium during the 48 hours' incubation. Typical figures obtained for these controls are given in Table I.

Table I.

	Mg. ammonia-N				Mg. urea- + ammonia-N			
Exp.	3	6	9	13	3	9	13	16
A	0·026	0·032	0·026	0·030	0·034	0·050	0·045	0·028
B	0·030	0·041	0·032	0·032	0·033	0·057	0·047	0·038
C	0·032	0·040	0·033	0·038	0·042	0·060	0·044	0·029
Total control	0·036	0·049	0·039	0·040	0·041	0·067	0·047	0·039

Figures for A and C are for 2 cc. of extract, figures for B are for 2 cc. of extract + two kidneys.

Each experiment consisted of a series of controls and growing preparations, in all of which the same medium and kidneys taken from embryos of the same litter were employed.

It will be seen that there is an increase of ammonia-N in the medium due to autolysis. No increase of urea was found. The amount of urea present in the kidneys is also extremely small, always within the limits of experimental error, so that sometimes no difference at all was obtained between the total urea- and ammonia-N figures and those for ammonia-N alone, occasionally small minus results even were obtained. It is therefore a matter of doubt as to

whether any urea is present or not in the embryonic kidney. The amount present in the embryonic extract was also very small, less than half that of the ammonia-N. The total amount of ammonia- + urea-N as estimated is of course outside the limits of experimental error, but the difference due to urea-N alone falls within that limit.

In addition to these controls, we devised a further control (D), which we have called the "resting tissue." This consists of the same amount of extract and tissue as the contents of the growth flasks, and like them is incubated for 48 hours. But by using a smaller flask for this resting tissue, we have prevented it from attaching itself to the surface of the glass, and thus also prevented growth from taking place. We have aimed at such a depth of fluid that, although the tissue is freely movable in it, it is only just submerged, and is able to obtain sufficient oxygen to prevent its death. It is obvious from the results of this resting tissue, compared with the total control (Table II), that autolysis has not taken place. We therefore concluded that the tissue was still living, as autolysates of adult mammalian kidneys form both ammonia and urea [McCance, 1924; Luck, 1924, 1]. It was, however, important to make sure of this point; so on one or two occasions at the end of 24 hours' incubation, the flask containing the resting tissue was opened, and part of the medium with some of the pieces of floating tissue was removed to a larger flask, and again sealed. The few pieces remaining behind in the original flask were stained and examined. No sign of growth could be detected. The new flask now contained such an amount of fluid that the remaining pieces of tissue could, if they were still living, attach themselves to the surface of the glass. After a further 24 hours' incubation they were stained and examined, and gave unmistakable evidence of growth, budding having taken place quite freely. This resting tissue having been proved to be living but non-growing tissue serves as a valuable control to the flasks in which growth has taken place. Any difference between ammonia- and urea-N in the growing and resting tissue, both having been subjected to the same conditions and compared with the "total control," can with some confidence be attributed to the actual growth of the tissue. The metabolic activity of the resting tissue seems to be reduced to an absolute minimum consistent with life, and may be described as its resting metabolism.

Table II. *Resting tissue compared with total control.*

	Resting tissue mg. ammonia-N + urea-N	Total control mg. ammonia-N + urea-N
Exp. 13 a	0·063	0·055
13 b	0·050	0·055
13 c	0·039	0·040
19	0·064	0·062

In view of the fact that the resting tissues must be considered to be living for at least the first 24 hours of the experiment we were interested to find a marked difference between these and the growing tissues as regards their activity in producing ammonia and urea. This has already been stated in a

preliminary paper [Holmes and Watchorn, 1927] and can therefore be discussed only very briefly here. In the case of the floating tissue the amounts of ammonia and urea after two days' incubation are exactly the same as the amounts contained in the total control. Examples are given in Table II, to which attention has already been drawn, and they show that the tissue has produced no ammonia or urea.

The growing tissue, on the contrary, produces considerable amounts of ammonia or urea or both together, as may be seen from the figures given in Table III. One might expect that, if this occurred to any extent during the life of the embryo, some sign of it would be found even in the floating tissues, but it must be remembered that the tissue has been allowed to become cold in the interval necessary for the preparation of the medium and the planting of the tissues. Cooling is very effectual in causing cessation of cell division in mammalian cells, and thus, if this production of ammonia and urea is associated with growth, as we believe it to be, it would only be found in cases where the growth had begun again.

In order to compare the results given by the resting and by the growing tissues, it was necessary to be certain that these latter had, in fact, grown. It was not, of course, possible to stain and examine preparations, and afterwards use them for estimation. We have sometimes resorted to the plan of growing special controls for staining, and we have sometimes been able to stain fragments of tissue which remained sticking to the glass after the vessel had been washed out several times for estimation purposes. As a general rule it is quite possible to detect growth with the aid of a binocular dissecting microscope without staining (the vessel merely being placed on black paper), and by this means it is possible to examine preparations in which the ammonia and urea are later to be estimated.

As it happens, in the case of embryo kidney tissue it has been found so consistently that tissues which are not growing form no urea or ammonia, that when these substances increase in amount, it may almost be assumed that growth has occurred. Floating preparations in which a few fragments of tissue have stuck to the glass and grown, show small increases in ammonia and urea. On the other hand, if a "growing" preparation fails to grow (this happened on two occasions, and the failure was eventually recognised as being due to an insufficiency of magnesium in the Ringer's solution), no increase in either of these substances is found.

The results of our experiments with "resting" kidney tissue agree well with those obtained by Warburg [1924] with sliced adult rat kidney tissue. He found that a considerable amount of ammonia appeared as the experiment progressed; but finding that this apparent production of ammonia by the kidney tissue had a very small temperature coefficient, he concluded that he was not dealing with a chemical process, but that the ammonia was merely being washed out of the tissue. This ammonia, which he considered to be existing preformed in the tissue, would be estimated in our tissue control B.

Table III.

Resting or control		Growing tissue		Increase	
Ammonia-N (mg.)	Urea-N (mg.)	Ammonia-N (mg.)	Urea-N (mg.)	Ammonia-N (mg.)	Urea-N (mg.)
0·036	0·011	0·075	0·034	0·039	0·023
0·036	0·011	0·058	0·012	0·022	Nil
0·039	0·029	0·070	0·023	0·031	Nil
0·055	0·022	0·096	0·039	0·041	0·017
0·055	0·022	0·060	0·055	Nil	0·033
0·058	0·015	*0·050	0·033	*Nil	0·018

* Poor growth.

It will be seen from the examples given in Table III that the amounts of ammonia and urea produced during growth are considerable, and far beyond the limits of the experimental error of this method. It is quite usual for the extra amount of ammonia produced to be equal to 0·1 % or more of the original wet weight. As examples the following experiments may be quoted:

Exp. 18 *c*. Wet weight 34 mg. Urea increased 0·033 mg.
Exp. 3 *a*. Wet weight 33 mg. Urea increased 0·023 mg.
Ammonia increased 0·039 mg.

We have no data as yet which might be helpful in showing to what chemical processes we must relate the production of these metabolites. Presumably proteins or similar substances (Carrel and Baker [1926] claim that, in the case of chick tissues growing *in vitro*, proteoses are the most important nutritive constituent of the medium) contained in the embryo extract must be broken down to their constituent amino-acids to be rebuilt into the cells of the growing tissue. Deamination of the amino-acids not required for the synthesis of cell proteins might then occur. If the deamination represents protein combustion for the purpose of obtaining energy for growth, we should be able to prevent this to a considerable extent by adding glucose to the medium, and this we propose to attempt shortly.

Nash and Benedict [1921] showed that during life the kidneys were capable of producing very considerable amounts of ammonia when the glomerular filtrate was acid. The mechanism of this ammonia production is not known. It was attempted at one time to account for it by assuming the presence of urease in the kidney, but no urease could be found when experiments were carried out *in vitro*. In our work we find no evidence for the existence of kidney urease, since the relative and absolute amounts of urea and ammonia present remain unchanged during 48 hours' incubation of our "resting" tissues.

There is, of course, no reason for assuming that the mechanism of this ammonia production is the same as the mechanism of its production by growing tissues. In fact in our growing tissues it seems that it may be urea or ammonia which is produced, or even both together (see Table III), and, according to the evidence of Bollman, Mann and Magath [1924] adult functioning kidney produces no urea. It should be pointed out that in the experiments of Bollman, Mann and Magath the kidneys were plentifully supplied

with glucose, which would tend to prevent urea formation if this originated in protein breakdown. We may further find that a supply of glucose to our growing tissues prevents the formation of urea in them also, or it may be that the metabolism of the growing tissue is in this respect quite distinct from that of the functioning adult tissue.

Experiments with brain tissue.

In addition to the experiments just described on kidney tissue, we have carried out the same technique using embryo brain tissue, and while we have not progressed far enough in this part of the work to allow any very definite conclusions to be drawn, one or two points seem to be quite well established and may be worth describing here, though later we hope to be able to deal with brain tissue in more detail.

In the case of brain tissue we have found it more difficult to obtain good growth, and to be certain when growth has occurred. In the experiments with kidney tissue we found it expedient, owing to the small size of the kidneys, to use fairly old embryos, and these gave very good growth. In the case of brain tissue, however, we found it more satisfactory to use young embryos, and even in these one cerebral hemisphere provides plenty of tissue.

The "resting" preparations gave results similar to those obtained with "resting" kidney tissue, in that the ammonia- and urea-N, taken together, showed no increase over the calculated amount in the total control, in fact, slight decreases were observed.

One remarkable fact was noticed, namely, that there was always some suggestion of an increase of ammonia at the expense of urea, that is, a suggestion of urease action. To obtain definite evidence of this, it is necessary to keep the filtrate from the ice-chest tissue control as cold as possible until it is required for estimations. Otherwise the urea in this control will also decrease in amount.

When such precautions are taken, results similar to those given in Table IV are obtained.

Table IV.

	Resting tissue (mg.)	Total control (mg.)
Ammonia-N	0·053	0·033
Urea-N	0·015	0·038
Ammonia-N + urea-N	0·068	0·071

So far the occurrence of urease in the tissues of higher animals has only been reported for the gastric mucosa of certain species [Luck, 1924, 2], but we have no reason so far to doubt that it is actually present in rat brain tissue, although probably only in small amounts. Adult rat brain tissue has been found to convert a little added urea to ammonia, but only a small number of experiments have been carried out. Rabbit brain tissue gave definitely negative results, showing no sign at all of the presence of urease. We have injected several rats subcutaneously and intraperitoneally with urea. With 1 g.

of urea the rats showed rigidity of the tail, twitchings and finally drowsiness, but with more urea (about 2 g. given at intervals) severe convulsions occurred about three-quarters of an hour later. So far as we know this effect has not been described for other animals known to contain no urease, as for example the rabbit; and while we cannot yet state that the effect we obtained in the case of the rat was due to the conversion of urea into ammonia, the possibility must be considered and further investigated.

In the case of the growing preparations we have obtained only a few satisfactory results, and these are most unlike those obtained with growing kidney tissue. When good growth has taken place we have usually found a considerable fall in the ammonia- and urea-N taken together. This suggests that some synthesis of nitrogenous substances at the expense of ammonia and urea must take place when the tissue is actively growing. More experimental data will have to be obtained, however, before this view can be expressed with certainty, and this work is now in progress.

Summary.

1. The technique of tissue culture has been employed to study the nitrogenous metabolism of growing tissues.

2. When growing, embryonic rat kidney tissue produces considerable amounts of ammonia and urea.

3. "Resting" embryonic kidney tissue, incubated under exactly the same conditions as the growing tissue, produces neither ammonia nor urea

4. Some preliminary experiments with brain tissue are described and the results contrasted with those obtained with kidney tissue. The probable existence of urease in the rat brain is discussed.

In conclusion we wish to express our indebtedness to Mr R. A. McCance, who was working with one of us (B. E. H.) for more than a year at this investigation when it was first undertaken, and to whom we owe many points of technique and many suggestions.

We wish also to thank Sir F. G. Hopkins for his kind interest in this work.

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XLVII. THE CATALYTIC ACTION OF TRACES OF IRON AND COPPER ON THE ANAEROBIC OXIDATION OF SULPHYDRYL COMPOUNDS.

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In a previous publication by the author [1924], it was shown that the rate of oxidation of glutathione and cysteine by the oxygen of the air was greatly reduced by the addition of traces of hydrogen cyanide. It was shown also that the rate of oxidation of glutathione and cysteine could be reduced to a similar low value by very careful purification in quartz vessels, taking all possible precautions to exclude iron; and further, that the rate of oxidation of the purified compounds could be accelerated by the addition of minute traces of iron. Evidence was given in support of Warburg's view that the inhibition by cyanide is due to the formation of a non-catalytic complex with traces of catalytic metals ordinarily present as impurities in the sulphydryl compounds.

During the course of these experiments, I found that not only the aerobic but also the anaerobic oxidation of sulphydryl compounds was greatly inhibited by small concentrations of hydrogen cyanide. In these anaerobic oxidations methylene blue acted as the hydrogen acceptor in place of oxygen. It seemed possible that, as in the case of the aerobic oxidation, the cyanide might be acting on traces of catalytic metal impurities. If this were true, these metals would be expected to catalyse not only the aerobic but also the anaerobic oxidation of sulphydryl compounds, and could not be regarded as acting as carriers or activators of oxygen as pictured by Warburg [1924]. It was therefore desirable to obtain samples of sulphydryl compounds and methylene blue as free as possible from traces of catalytic metals, and then to show that the rate of anaerobic oxidation could be accelerated by the addition of traces of iron to the purified compounds.

The present paper contains the results of these experiments. Most of these results were obtained in 1924, and a brief reference to them was made in a paper by Thurlow [1925]. In 1926, a paper appeared by Toda [1926], containing experiments similar to some of those which I am about to describe.

Both his and my own experiments yielded a similar result, and he draws the same conclusion as was indicated in the reference to my experiments by Thurlow [1925].

Inhibition by hydrogen cyanide.

The following experiments show the great inhibition produced by traces of hydrogen cyanide on the reduction of methylene blue by cysteine, glutathione and thioglycollic acid. In this group of experiments, the vacuum tubes and technique described by Thunberg were used. After twice evacuating and filling with nitrogen, the tubes were again evacuated and immersed in a water-bath, the latter being mechanically stirred and maintained at 37°.

The vacuum tubes each contained 0·3 cc. of methylene blue (1 part in 5000 of water) and a solution of the particular sulphydryl compound neutralised with dilute sodium hydroxide. 0·1 cc. of potassium cyanide solution (previously neutralised with HCl) was added to one tube and an equal volume of water to the other tube. The total volume of solution in each tube was made up to 2·0 cc. with phosphate buffer.

Table I.

Sulphydryl compound	p_H	Time in min. for decoloration of Mb
Cysteine (0·3 mg.)	7·0	Without cyanide 12 min.
,,	,,	+ *M*/4000 HCN 42
,,	7·0	Without cyanide 12
,,	,,	+ *M*/8000 HCN 27
,,	7·4	Without cyanide 12
,,	,,	+ *M*/2000 HCN 45
,,	7·6	Without cyanide 10
,,	,,	+ *M*/2000 HCN 60
Glutathione (1·2 mg.) ...	7·0	Without cyanide 35
,,	,,	+ *M*/2000 HCN 110
Thioglycollic acid (1·2 mg.)	7·4	Without cyanide 95
,,	,,	+ *M*/4000 HCN 240

From these experiments it is evident that the addition of very small quantities of cyanide produces a marked inhibition in the rate of reduction of methylene blue by sulphydryl compounds.

On adding iron to solutions containing cyanide, it was found that the inhibition produced by the latter was considerably reduced. On adding iron to the solutions of sulphydryl compounds in the absence of cyanide, the rate of reduction was increased, though this increase was not very marked, owing to the relatively large amount of iron already present in the solutions.

Preparation of iron-free methylene blue.

The method used for preparing iron-free methylene blue was similar to that previously employed by the author in purifying cysteine [Harrison, 1924]. It depends, firstly, on the precipitation of the iron by hydrogen sulphide in presence of baryta, and, secondly, on crystallisation of the leuco-compound from pure alcohol. In view of the fact that cysteine forms a compound with

iron while methylene blue shows no such tendency, it seemed likely that the method would give even better results than with cysteine.

Two grams of crystalline methylene blue (the free base) were added to 150 cc. of water in a Pyrex conical flask which had been previously boiled out with pure concentrated hydrochloric acid. 20 g. of finely powdered crystalline baryta were then added and, after shaking, a rapid stream of well-washed hydrogen sulphide was passed for half an hour. The methylene blue was reduced to the leuco-compound which, together with the iron, was thrown down as a greyish precipitate. This was allowed to stand for 24 hours in a closed vessel, and then shaken with 100 cc. of moist ether which had been previously distilled from quartz vessels and saturated with hydrogen sulphide. The ether dissolves most of the leuco-compound, leaving the iron behind as a greenish basic sulphide. After standing for 2 hours the greater part of the ether layer was carefully poured into a quartz flask, shaken with a few cc. of pure water to remove any traces of barium, and poured off into an acid-extracted filter paper resting in the neck of a quartz flask. After filtering, the mouth of the flask was covered with a filter paper and the ether was distilled off, leaving the pale yellow leuco-methylene blue. This was dissolved in the minimum quantity of hot alcohol (distilled from quartz and containing a little dissolved hydrogen sulphide), half the volume of pure water was added and the solution was cooled under the tap for a short time.

Crystalline leuco-methylene blue separated out in fine needles and was filtered in a quartz Gooch crucible lined with an acid-extracted filter paper, and washed several times with 70 % alcohol (which had been distilled in quartz). If care be taken in the preceding operation to avoid undue exposure to the air, the leuco-methylene blue is obtained quite free from the oxidised form. After drying on the pump for a few minutes, the leuco-compound started to oxidise rapidly. The drying was completed in a vacuum desiccator over sulphuric acid.

The yield obtained under these conditions is small, but may be increased, though at the possible sacrifice of purity, by crystallising from more dilute alcohol.

Purification of sulphydryl compounds.

Cysteine hydrochloride was rendered free from traces of iron by the method employed in experiments on the aerobic oxidation of cysteine by Sakuma [1923] and Harrison [1924].

Thioglycollic acid was purified from traces of metallic impurities by distillation in hydrogen at 16 mm. Hg pressure, using quartz vessels.

A large number of experiments was then carried out on the rate of reduction of the pure methylene blue by the pure sulphydryl compounds. Preliminary experiments were carried out in quartz vacuum tubes, but it was found difficult to obtain good agreement between duplicates, possibly owing to the presence of oxygen adsorbed on the walls of the quartz tubes. It was

found, however, that by subjecting glass vacuum tubes to a thorough cleaning with acids before each experiment, contamination of the solutions by traces of catalytic metals did not occur, and that the difficulties associated with the use of quartz vacuum tubes could be obviated.

Owing to variations in the minute quantities of metals remaining in different samples of the purified compounds and to the difficulty of ensuring that absolutely no metallic impurities are introduced during the making up of the solutions, the reduction times observed in different experiments employing the same quantities of sulphydryl compound and methylene blue varied somewhat. Another possible source of this variation may be the differences in the intensity of illumination of the water-bath on different days. Preliminary experiments appear to indicate that sunlight accelerates the rate of reduction of methylene blue by cysteine and thioglycollic acid, and Dixon and Tunnicliffe [1923] observed a similar effect in the case of glutathione. Nevertheless, these facts do not in any way affect the conclusions drawn in this paper on the relative rates of anaerobic oxidation of sulphydryl compounds in the presence and absence of catalytic metals, for in every experiment duplicate tubes were employed and these duplicates almost invariably showed good agreement. In the few cases where good agreement was not obtained, the results were discarded and the experiments repeated.

Before each experiment, the glass tubes were treated with small quantities of a mixture of concentrated nitric and sulphuric acids to which a few drops of alcohol were cautiously added. After repeating this treatment, the tubes were boiled out with pure concentrated hydrochloric acid and allowed to stand filled with concentrated hydrochloric acid. Finally the tubes were well rinsed out with quartz-distilled water. After this treatment the tubes were found to be perfectly satisfactory and to give good duplicates. The lower part of the ground glass stoppers was left ungreased to avoid possible contamination of the solution from the tap grease. After dissolving the pure sulphydryl compound in quartz-distilled water, the solutions were brought to the required p_H by the addition of dilute ammonium hydroxide previously distilled in quartz vessels. The alkali was added from a quartz burette, the volume required being determined by separate titration. No buffer was used in the experiments with the purified compounds. The tubes were evacuated with the water-pump and filled with nitrogen (washed with alkaline pyrogallol). This was repeated, and finally the tubes were evacuated and completely immersed in the water-bath, the side tubes being first filled with water to reduce the chance of leakage. The p_H was checked at the end of each experiment by means of Clark and Lubs' standard indicators. A few drops of the indicator previously boiled to expel any dissolved air were introduced into the side tube of each reduction tube and allowed to mix with the solution inside the tube by partially turning the stopper, care being taken to prevent the entry of any air. In all experiments described in this paper, 0·3 cc. of purified methylene blue (1 part dissolved in 5000 parts of quartz-distilled water) was used in each tube. The total volume

of solution in each tube was always made up to 2·0 cc. In all experiments employing iron, a freshly prepared solution of ferric chloride was used. The copper used was added as copper sulphate solution. The results quoted in the following tables are those of typical experiments chosen from a large number. Each experiment was carried out in duplicate.

Table II shows the catalytic effect of traces of iron on the anaerobic oxidation of purified cysteine at 37°.

Table II.

Each tube contained 0·4 mg. of purified cysteine hydrochloride. Iron added as ferric chloride. Temp. 37°.

No.	p_H	Iron added (mg.)	Time in min. for decoloration of Mb
1	7·3	—	18 min.
	7·3	0·01	9
2	7·0	—	53
	7·0	0·001	24
3	7·7	—	23
	7·7	0·001	15

It was found that by carrying out the experiments at 25° instead of at 37° the rate of decoloration of methylene blue was slower, and that consequently differences in reduction time could be measured more easily. All the experiments subsequently to be described were therefore carried out at 25°. Table III shows the catalytic effect of traces of iron on the anaerobic oxidation of purified cysteine at 25°.

Table III.

Iron added as $FeCl_3$. Temp. 25°.

No.	Cysteine-HCl mg.	p_H	Iron added mg.	Reduction time of Mb in min.
1	0·4	8·5	—	90
	,,	,,	0·001	55
2	0·5	8·4	—	39
	,,	,,	0·001	18
3	0·8	7·8	—	98
	,,	,,	0·001	33
4	0·8	7·6	—	50
	,,	,,	0·001	14
5	0·8	5·6	—	73
	,,	,,	0·001	57

Table IV.

Each tube contained 0·8 mg. of purified cysteine hydrochloride. Copper added as $CuSO_4$. Temp. 25°.

No.	p_H	Cu added mg.	Reduction time of Mb in min.	Reduction time of Mb with HCN
1	7·6	—	50	—
	,,	0·0001	9	—
	,,	0·001	$<\frac{1}{2}$	—
2	7·4	—	66	+ M/1000 HCN 65 min.
	,,	0·0001	8	—
	,,	0·0002	6	—
3	7·6	—	60	+ M/500 HCN 72 min.
	,,	0·00001	26	—
	,,	0·00005	9	—

It is evident from Tables II and III that iron acts as a powerful catalyst in the anaerobic oxidation of cysteine, both on the acid and alkaline side of neutrality.

Table IV shows the very powerful catalytic effect of minute traces of copper on the anaerobic oxidation of purified cysteine.

For purposes of comparison, it may be mentioned that a sample of ordinary unpurified cysteine hydrochloride (0·8 mg.) in p_H 7·6 phosphate buffer under the same experimental conditions as in Table IV reduced the methylene blue in 10 minutes in the absence of cyanide, while in presence of $M/1000$ HCN, it reduced in 70 minutes; on the other hand it will be seen from *Exp.* 3 in Table IV that under similar conditions 0·8 mg. of *purified* cysteine hydrochloride reduced the methylene blue in 60 minutes without HCN, and in 72 minutes after the addition of $M/500$ HCN. From Table IV there is seen to be very little, if any, inhibition produced by HCN on the rate of oxidation of the purified cysteine. From the results in Table IV it is evident that a quantity of copper so minute as five millionths of a milligram per cc. produces a considerable acceleration in the anaerobic oxidation of cysteine. By comparison with Table III, it will be seen that iron is considerably less active as a catalyst than copper. Nevertheless, five ten-thousandths of a milligram of iron per cc. was found to produce a considerable acceleration.

A number of experiments were then carried out using purified thioglycollic acid instead of cysteine and it was found that in marked contrast to cysteine, the rate of reduction of methylene blue by purified thioglycollic acid was not appreciably increased by the addition of iron in quantities of the same order as those employed in the experiments with cysteine. On the other hand, a trace of copper was found to act as a very powerful catalyst in the anaerobic oxidation of purified thioglycollic acid. This is shown in Table V.

Table V.

Copper added as $CuSO_4$. Temp. 25°.

No.	Thioglycollic acid (mg.)	p_H	Cu added (mg.)	Reduction time of Mb in min.
1	1·2	7·4	—	137
	,,	,,	0·001	$<\frac{1}{2}$
	,,	,,	0·0005	10
2	1·6	7·6	—	35
	,,	,,	0·001	3
3	1·2	7·9	—	155
	,,	,,	0·001	6

The addition of $M/1000$ HCN to the purified thioglycollic acid was found to produce practically no inhibition, though the oxidation of a solution of the pure thioglycollic acid to which a minute trace (0·0005 mg.) of copper had been added was markedly inhibited by $M/1000$ HCN.

In investigating the effect of iron on the anaerobic oxidation of purified thioglycollic acid, a slight acceleration appeared to be induced by relatively large quantities of iron, but in view of the extremely powerful effect of copper on this oxidation, it seems possible that this effect observed with larger con-

centrations of iron may be due to traces of copper (or possibly some other catalytic metal) present in the iron solutions. Owing to the non-sensitivity of thioglycollic acid to iron, and to its ready purification by a single distillation in quartz, it is easy to obtain solutions of this compound the rate of oxidation of which is not appreciably inhibited by the addition of HCN. On the other hand, such solutions of cysteine are more difficult to obtain.

There seems little reason to believe that the action of catalytic metals on the anaerobic oxidation of pure glutathione would be essentially different from their action on pure cysteine, for in the experiments on the aerobic oxidation of purified glutathione, it was shown that traces of iron produced a similar catalytic effect on the purified glutathione to that produced on purified cysteine [Harrison, 1924]. Further, it is shown in the present paper that the anaerobic oxidation of glutathione is inhibited by traces of cyanide in a similar way to that of cysteine and thioglycollic acid. It seems highly probable, therefore, that the anaerobic oxidation of pure glutathione is catalysed by traces of metals in a similar way to that of cysteine.

Discussion.

In a recent paper by Moureu, Dufraisse and Badoche [1926], it is suggested that the inhibitions caused by hydrogen cyanide are not necessarily to be explained by the inactivation by cyanide of traces of catalytic impurities, but that the facts can equally well be explained by the general "antioxygen" theory of Moureu and Dufraisse [1926]. According to this theory, the substance which is oxidising and the substance which is causing inhibition both take up oxygen to form compounds of the peroxide type. The interaction and mutual decomposition of the two peroxides then takes place. This leads to the regeneration of the original unoxidised substances, and thus oxidation is prevented. These authors suggest that the inhibition caused by hydrogen cyanide in the oxidation of sulphydryl compounds can be explained in the above manner, but that such is not the case is evident from the following considerations. Firstly, the more carefully the sulphydryl compounds are purified from traces of catalytic metals, the less becomes the inhibition produced by cyanide [Harrison, 1924]. Secondly, pyrophosphates have been shown to inhibit the oxidation of sulphydryl compounds in a similar way to cyanides, and in this case the formation of a transitory peroxide of the inhibitor (as required by the antioxygen theory) can hardly be taking place. Finally, the experiments described in the present paper show that the inhibition by cyanide also occurs in the *anaerobic* oxidation of sulphydryl compounds, a reaction in which formation of peroxides of the substance oxidising or of the inhibitor can scarcely be regarded as possible.

From the results of the experiments given so far in this paper, it is evident that the anaerobic oxidation of the sulphydryl group is accelerated by traces of iron in a similar way to its aerobic oxidation. In the experiments on the anaerobic oxidation, methylene blue replaces oxygen as the hydrogen acceptor.

Since iron produces a similar catalytic effect on both the aerobic and the anaerobic oxidations of the sulphydryl group, it would appear that the iron cannot be acting as an activator of oxygen as suggested in Warburg's theory. It would seem rather that the experimental facts can be satisfactorily explained by assuming that in both the aerobic and anaerobic oxidation of the sulphydryl compounds, the iron (or other catalytic metal) is acting as an intermediary catalyst, becoming alternately oxidised and reduced. Ferric iron is reduced by the sulphydryl compound; the ferrous compound thus formed then reduces the methylene blue to methylene white in the anaerobic experiments, or oxygen to hydrogen peroxide in the aerobic experiments. The resulting ferric compound is then again reduced by a further quantity of the sulphydryl compound, so bringing about the oxidation of the latter. The essentials of the process may be expressed as follows:

$$(1)\quad 2Fe(OH)_3 + 2RSH \rightarrow RSSR + 2H_2O + 2Fe(OH)_2$$

$$(2)\quad 2Fe(OH)_2 + 2H.OH \rightarrow 2Fe(OH)_3 + 2H$$

$$(3)\quad (a)\ 2H + \begin{matrix} O \\ \| \\ O \end{matrix} \rightarrow \begin{matrix} H{-}O \\ | \\ H{-}O \end{matrix}$$

$$(b)\ 2H + Mb \rightarrow MbH_2$$

Reaction (2) will proceed in a forward direction in the presence of a hydrogen acceptor such as oxygen or methylene blue, so long as the reduction potential of the system expressed by Fe··/Fe··· is sufficiently high to reduce the hydrogen acceptor.

This condition is ensured by the re-reduction of the ferric iron by the excess of sulphydryl compound in the solution, the reduction potential of the system Fe··/Fe··· thus being maintained. Dixon [1927] has recently described experiments which indicate that the reduction potential of the sulphydryl system is considerably higher than that indicated by previous work. The sulphydryl compounds will tend to maintain the reduction potential of the ferrous-ferric system at a value well above that of the system MbH_2/Mb, and the reduction of the methylene blue will proceed virtually to completion. In such a mechanism, the velocity of oxidation of the sulphydryl compound will be determined by the slower of the two reactions, namely, the reduction of the ferric compound by the sulphydryl group, and the subsequent reduction of the hydrogen acceptor by the resulting ferrous compound. A positive catalysis by iron will occur provided that the slower of these two reactions is more rapid than the direct reduction (without the intervention of iron) of the hydrogen acceptor by the sulphydryl compound.

Such a conception of iron as an intermediary catalyst does not appear to necessitate the assumption of an activation of oxygen by iron as pictured by Warburg. It would appear rather that molecular oxygen is acting as an acceptor for hydrogen formed in reaction (2). It is probable that the catalysis proceeds by the alternate oxidation and reduction of an iron-sulphydryl complex rather than by the free inorganic iron indicated in reactions (1) and (2). There is abundant evidence for the existence of this complex. The

assumption of its formation, while it does not modify in any essential the theory outlined above, will explain the non-precipitation of ferric hydroxide from the reacting system during the catalysis in alkaline solution. There seems no reason for assuming an activation of oxygen by iron in the aerobic system any more than we assume an activation of methylene blue by iron in the anaerobic system. The experimental results given in the present paper combined with previous work on the subject indicate that there is no essential difference between the catalysis in the aerobic and in the anaerobic systems, and the mechanism outlined above would explain the catalysis in the two systems on exactly similar lines. A somewhat similar explanation of the catalysis by iron of the oxidation of hexoses in phosphate solution has recently been suggested by Blix [1927].

An alternative possible explanation of the catalysis of the oxidation of sulphydryl compounds is one similar to that suggested by Toda [1926], namely, the activation of the hydrogen of the sulphydryl group by iron. In the formation of the complex between iron and the sulphydryl compounds, the hydrogen of the sulphydryl group is assumed to be activated, and this hydrogen is then readily taken up by methylene blue or oxygen. It would seem, however, that the theory of the alternate oxidation and reduction of the catalytic metal is the more attractive explanation of the facts for several reasons. Firstly, it does not involve the assumption (for which as yet there is no direct evidence) of an activation by iron of the hydrogen of the sulphydryl group. Secondly, there is definite evidence that an alternate oxidation and reduction of iron takes place during the catalytic oxidation of the sulphydryl group. The change of ferrous to ferric iron and *vice versa* can actually be observed by the appearance and disappearance of the violet colour of the ferric-cysteine complex in the alkaline oxidising solution [Harris, 1922], and it was shown by Harrison [1924] that a similar colour change occurs also in glutathione solutions oxidising in the presence of iron. Further, it appears significant in this connection that the metals which were found by Mathews and Walker [1909] to catalyse the oxidation of cysteine are all capable of existing in at least two well-defined different states of valency. The evidence given in favour of the oxidation-reduction theory of catalysis by iron does not, however, exclude the possibility that the direct activation of the hydrogen of the sulphydryl group by iron may also be playing a part. The two mechanisms may be going on side by side.

The experiments described in the present paper, while showing the enormous catalytic effect of certain metals, at the same time appear to indicate that the reduction of methylene blue by sulphydryl compounds can take place with a not inconsiderable velocity, even in the absence of catalytic metals. In the purest solutions of methylene blue and sulphydryl compounds obtained, it was found that the addition of cyanide produced no appreciable inhibition in the rate of anaerobic oxidation. Any traces of catalytic metals remaining in the purified solutions would presumably be inactivated by the

cyanide, and yet, even in the presence of cyanide, the velocity of the reaction was found to be practically unaltered. It is of interest to compare this result with the results of experiments obtained by the author [Harrison, 1924] in the case of the aerobic oxidation of glutathione and cysteine. It was found in these aerobic experiments that cyanide did indeed produce an inhibition in the rate of oxidation of the purified compounds, though, even in the presence of excess of cyanide, an appreciable oxidation of the sulphydryl compounds still occurred. It seems not unlikely, therefore, that the sulphydryl group can oxidise to some extent, even in the absence of catalytic metals, and that these metals act by accelerating a reaction which is already taking place at a measurable rate.

Oxidation of iron-free methylene white.

According to Warburg, molecular oxygen can under no circumstances act as an acceptor for hydrogen; the molecular oxygen must first be activated by combination with iron. If this view be correct, it follows that the aerobic oxidation of any substance by loss of hydrogen cannot take place in the complete absence of iron. However, during the preparation of iron-free methylene blue (as described above), it was interesting to find that the iron-free leuco-compound oxidised rapidly on exposure to the air. Here was an example of a substance containing two labile hydrogen atoms which could be taken up by the molecular oxygen of the air, even in the absence of iron. The oxidation was evidently not brought about by traces of iron introduced from the air during the drying of the leuco-compound on the pump, for small quantities of leuco-compound which remained behind in the quartz flask were similarly oxidised.

Further evidence for the ability of molecular oxygen to act as a hydrogen acceptor for the labile hydrogen of leuco-methylene blue is afforded by the following experiments.

To each of two vacuum tubes were added 0·1 cc. of cysteine solution (containing 0·3 mg. of free cysteine), 0·3 cc. of 1/5000 methylene blue and 1·5 cc. of p_H 7·6 phosphate buffer. 0·1 cc. of $M/20$ KCN (previously neutralised with HCl) was then added to one tube and an equal volume of water to the other tube. The tubes were then evacuated and immersed in a water-bath at 37° until the methylene blue was completely decolorised in both tubes. The tubes were then opened to the air and shaken. It was found that the solutions in both tubes became slightly blue almost immediately, and the rate of progressive deepening was the same in both tubes. The tube to which cyanide had been added smelled strongly of HCN at the end of the experiment. In another experiment, 10 cc. of a 1/5000 solution of iron-free methylene blue in pure water was reduced by passing a current of pure hydrogen sulphide (prepared from Sb_2S_3) through the solution for half an hour. The solution was then divided into two equal parts in two quartz test tubes. To one tube was added 0·1 cc. of $M/20$ KCN (carefully neutralised with HCl) and to the

other was added an equal volume of pure water. The tubes were then evacuated on the pump to remove excess of hydrogen sulphide, and, on opening to the air and shaking, the rate of oxidation of the leuco-methylene blue was again observed to be the same in both tubes. Traces of iron impurities in the solution would have been inactivated by the hydrogen cyanide, and since the rate of oxidation was the same, either in the presence or in the absence of cyanide, it is evident that iron is not necessary for the oxidation of leuco-methylene blue, but that the latter can give up hydrogen directly to unactivated molecular oxygen.

This fact is of importance in considering the validity of Warburg's theory. Warburg states that molecular oxygen is entirely unable to act as an acceptor for hydrogen unless first activated by iron. Nevertheless, the above experiment proves that molecular oxygen is capable of combining with the hydrogen of leuco-methylene blue, even in the complete absence of iron. It would appear, therefore, that in certain oxidations at any rate, molecular oxygen *can* function directly as a hydrogen acceptor. It is true that the oxidation of leuco-methylene blue is not actually a biological reaction, but there seems no reason why it should be regarded as essentially different in mechanism from the oxidation of a biological system. As shown by Dixon and Thurlow [1925], the aerobic oxidation of hypoxanthine in the presence of the xanthine oxidase is also unaffected by the addition of cyanide. It would therefore seem that both in the oxidation of leuco-methylene blue and in the oxidation of hypoxanthine in the presence of xanthine oxidase, labile hydrogen is taken up by molecular oxygen without the intervention of iron. Warburg [1924], however, refused to concede that the oxidation of hypoxanthine in the presence of xanthine oxidase is a tissue respiration system. It is difficult to understand why this reaction should not be classed as a part of tissue respiration, since the oxidation of hypoxanthine is known to occur in the tissues.

Summary and Conclusions.

Briefly, the chief conclusions that may be drawn from this paper are as follows. The fact that hydrogen cyanide inhibits not only the aerobic but also the anaerobic oxidation of sulphydryl compounds indicates that it is not inhibiting an oxygen activator. Such a fact means that inhibition by cyanide in an oxidising system does not justify the assumption that oxygen activation is a necessary process in that system. The observation that the addition of very small traces of certain metals to purified sulphydryl compounds accelerates not only the aerobic but also the anaerobic oxidation of the sulphydryl group affords further indication that the catalytic action of these metals is not that of an oxygen activator. It is suggested that this catalysis of the oxidation of the sulphydryl group by metals is effected by the alternate oxidation and reduction of the catalytic metal by means of which hydrogen is made available for acceptance by molecular oxygen or by methylene blue. Such an explanation makes comparable the mechanism involved in the catalysis of both the

aerobic and the anaerobic oxidation of the sulphydryl compound. These experiments indicate the important part played by extremely minute traces of iron and other metals in biological oxidations involving the sulphydryl group. Copper, in the concentrations shown to be effective, may well be regarded as a physiological factor, since small quantities of copper have been shown to be present in many living tissues. Another possible function of small quantities of iron has been indicated by Harrison and Thurlow [1926] in whose experiments it was shown that in the presence of the xanthine oxidase system to which ferrous iron had been added, the oxidation of certain physiological substances such as lactic acid and β-hydroxybutyric acid could be brought about. In this system, the iron appears to be acting as a peroxidase rather than by the mechanism considered above.

The experiments in the present paper indicate that the reduction of methylene blue by sulphydryl compounds may take place to an appreciable extent even in the complete absence of catalytic metals. A similar result was obtained in an earlier paper in the aerobic oxidation of sulphydryl compounds. Further evidence against the necessity for oxygen activation in this system is thus afforded. The fact that iron-free leuco-methylene blue has been shown to be strictly autoxidisable affords another example of the direct acceptance of hydrogen by molecular oxygen.

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XLVIII. NOTE ON AN EFFICIENT GAS SCRUBBER.

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(*Received February 28th, 1927.*)

The scrubber described and illustrated below is particularly suitable for the analysis of a volatile substance which is based on volatilising the compound and trapping it in a suitable liquid. The author has used the scrubber with advantage in the method of estimating small amounts of alcohol in body fluids as described by Cannan and Sulzer, and also in the estimation of urea in blood by Van Slyke's method. Doubtless it could be used for other similar methods.

The efficiency of the scrubber depends upon the incorporation of one of the well-known glass filter plates made by the famous Schott firm of Jena. All the gas or mixture of gases and vapours has to pass through the pores of the plate and must thus emerge as tiny bubbles (see Fig. 1). Not only is it necessary to get efficient contact between the trapping liquid and the gas mixture, but there must also be an efficient circulation of the former through the inside of the bubbler above the plate. The ideal method of effecting this is to have a small tube through the centre of the porous plate coming up from the bottom of the bubbler. Messrs Schott kindly made some of these to the writer's design some eighteen months ago. One of them is illustrated in Fig. 1, which shows not only the bubbler but also the holder and a ground-on glass container for the trapping liquid. Two of these containers are ground to the same holder and so can be used alternatively to save time. The mixture of gases is drawn through the apparatus in the direction indicated by the arrows. The upward motion of the bubbles above the porous glass plate results in liquid being sucked up through the small centre tube which pierces the plate.

It will be noted that the upper end of the bubbler tube above the plate is just turned down again. This stops splashing and gives another washing (were any necessary) if the level of this turned down end just touches the liquid; this can easily be adjusted if the same volume of a standard liquid is used in the container each time. The advantages of this small piece of apparatus, as illustrated, may be summed up as follows.

1. Thoroughly efficient scrubbing.
2. Thorough circulation of the trapping liquid.
3. Prevention of splashing of the latter up and through the exit tube.
4. It is made entirely of glass. There is thus no possibility of the liquid coming into contact with oxidisable material such as rubber.

5. It can be easily washed out at the end of an experiment, the plate itself and the inlet tube being readily flushed out by connecting the end *A* to a distilled water supply giving a pound or two of pressure.

6. Its relative cheapness—the whole apparatus[1], including an extra container and the outside vaporising tube (which is not shown in the illustration) costing about 45*s*.

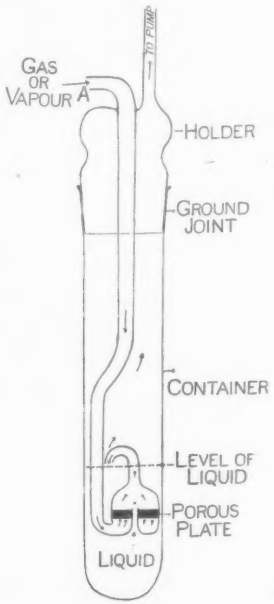

Fig. 1.

[1] Made for me by the Scientific Glass Blowing Company of 12 Wright Street, Oxford Road, Manchester, who keep a stock of these Jena bubblers made to my design.

XLIX. DECOMPOSITION OF HEXOSEPHOSPHATES BY *B. COLI COMMUNIS*, ESCHERICH.

By RODGER J. MANNING.

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(*Received February 25th, 1927.*)

Both hexosemonophosphoric and hexosediphosphoric acids are decomposed by *B. coli communis*, Esch. with production of acid and gas. The following experiments were made to ascertain whether the products of decomposition of these acids differed in any marked way from those formed by the action of the same organism on glucose and fructose. The barium salts of the acids were prepared by the method described by Robison [1922].

On account of the toxicity of the barium salts, as well as of the slight solubility of the barium hexosediphosphate, the sodium salts were formed and used in the experiments. These were decomposed by *B. coli communis* in atmospheres of oxygen and of nitrogen, respectively, at 37° for periods of seven to ten days and the resultant liquids then analysed. The products of decomposition were found to be carbon dioxide, alcohol, formic, acetic, lactic and succinic acids, whether the decomposition took place in presence or absence of oxygen[1], this being in agreement with the observations of Grey, when decomposing glucose by *B. coli communis* [1919, 1920; Grey and Young. 1921].

The more rapid the bubbling of oxygen through the bacterial digest the greater was the amount of the sugar decomposed completely to carbon dioxide. In the anaerobic digest the amounts of alcohol and of all the acids were markedly greater than in the aerobic, the carbon dioxide being correspondingly less.

Purification of barium hexosediphosphate.

256 g. of crude barium hexosediphosphate were carefully triturated with 2560 cc. water and filtered. The residue was again triturated twice in succession very carefully with 400 cc. water, filtered and the combined filtrates were used for the preparation of the barium hexosemonophosphate. The residue containing insoluble inorganic phosphates and barium hexosediphosphate was now ground up carefully over a period of two hours with 100 parts of water and from this solution by twice precipitating with lead acetate as described by Robison [1922] the purified barium hexosediphosphate used in the experiments was finally obtained.

[1] No estimation of gaseous hydrogen was made.

Method of experiment.

Ten grams of barium hexosediphosphate were ground up with 4·7 g. of sodium sulphate in 250 cc. water. The barium sulphate was filtered off and washed with 50 cc. water. The combined washings and filtrate were now sterilised by filtration through a Chamberland candle and to the solution of sodium hexosediphosphate thus obtained the bacterial suspension was added. To prepare this 2 cc. of a 24-hour bouillon culture of *B. coli* were added to each of ten Roux bottles and incubated for two days. The bacteria were then washed off by using 500 cc. of a sterile solution containing 6 g. potassium sulphate and 0·5 g. magnesium sulphate per litre. Filtered oxygen was bubbled slowly into the liquid contained in a 1500 cc. flask to which was connected an absorption tube containing alkali for the retention of carbon dioxide, and the whole kept at 37° for ten days. At the end of that time washed air was bubbled through vigorously for four hours and the analysis carried through as subsequently described.

In the case of the anaerobic fermentation of the sodium hexosediphosphate, nitrogen was bubbled through the liquid in the digestion flask for two hours before the carbon dioxide absorbing apparatus was attached. The outlet of this was placed below mercury and the whole left in a warm room for one week. At the end of the fermentation period nitrogen was passed through the apparatus for four hours with the delivery tube of the nitrogen well below the surface of the liquid.

Analytical methods.

Carbon dioxide. In determining the carbon dioxide formed, the amount absorbed by the alkali in the absorbing apparatus was determined by double titration, using methyl orange and phenolphthalein. Some carbon dioxide was at times retained in the combined form in the decomposition mixture as carbonate or bicarbonate; this residual carbon dioxide was determined by taking a portion of the bacterial digest, strongly acidifying with sulphuric acid and aspirating for a couple of hours into alkali.

For the determination of the alcohol and volatile acids, formic and acetic, about 500 cc. of the bacterial digest were acidified with 10 cc. phosphoric acid and distilled with steam. The volatile acids passed over quite slowly so that it was necessary to distil as much as two or three litres, titrating each successive portion of 250 cc. until a small but almost constant titre was obtained, due doubtless to the lactic acid that continued to come over in minute quantities. The separation of the volatile and non-volatile acids was therefore not very accurate.

Alcohol. Part of the neutralised distillate was used for the determination of the alcohol; 50 cc. were usually taken, 25 cc. distilled off and the alcohol was estimated in the distillate by the method of Pringsheim [1908]. Care was taken to keep the final volume of the sample oxidised by the dichromate constant and equal to that used in standardising the dichromate, otherwise

the readings were quite irregular. A dilution of 110 cc. was used throughout and at that dilution 1 cc. $N/20$ dichromate was found to oxidise 0·5919 mg. alcohol. As traces of alcohol were sometimes retained by the barium hexosediphosphate and barium monophosphate, the alcohol content of these was estimated by dissolving the barium salts in water or decomposing them by means of sodium sulphate, filtering, distilling and estimating the alcohol in the distillate. A little alcohol was sometimes carried over from the digest into the carbon dioxide absorbing apparatus and this also had to be estimated.

Volatile acids. The total neutralised steam distillate was evaporated to dryness on a water-bath and extracted with water and filtered from various wax-like substances invariably present. The formic acid was estimated in this extract by the method of Blank and Finkenbeiner [1898] and the acetic acid by difference from the total titre.

Succinic and lactic acids. These were estimated by the method described by Grey [1917].

Carbohydrate. Any residual reducing sugar left at the end of the bacterial digest was estimated by precipitating the protein from a sample of the digest with a slight excess of Patein's mercuric nitrate solution, and using Bertrand's method to determine the hexose in the clear filtrate. Usually no residual carbohydrate was found in aerobic fermentation unless the oxygen supply was limited, but under anaerobic conditions as much as 10 % of the hexose was still unfermented, although the hydrolysis of the sodium hexosediphosphate had been nearly complete. The carbohydrate was found not to be a free hexose for the most part but rather a non-reducing polysaccharide, which was estimated by taking 20 cc. of the filtrate, acidifying with 4 cc. dilute sulphuric acid, heating in a boiling water-bath for 1–2 hours, neutralising, and then determining the hexose by Bertrand's method.

Inorganic and organic phosphorus. The phosphorus present both as free phosphate and combined in the hexosephosphates was determined by Briggs' modification of the Bell-Doisy method [1922]. This was best done by adding 5 cc. of an 8 % solution of trichloroacetic acid to 10 cc. of the digest and filtering. 10 cc. of the clear filtrate were diluted to 100 cc. and this solution was used for inorganic and organic phosphorus determinations.

During the process of bacterial digestion both aerobically and anaerobically, practically all the hexosephosphates were decomposed so that only a small amount of the phosphorus was found not to be present as inorganic phosphate. From the increase in the inorganic phosphate, the amount of the hexosephosphate that had been decomposed was calculated and from this the carbon for the carbon balance.

Products of decomposition of sodium hexosediphosphate by B. coli.

The amounts of the various substances formed by aerobic and anaerobic decomposition were as follows:

Table I.

	Aerobic decomposition		Anaerobic decomposition	
	Weight g.	C atoms per mol. of sugar fermented	Weight g.	C atoms per mol. of sugar fermented
Carbon dioxide ...	3·1930	5·62	0·5632	1·26
Alcohol	0·0900	0·30	0·2393	1·03
Acetic acid	0·0579	0·15	0·5860	1·92
Formic acid	0·0021	0·003	0·1352	0·29
Lactic acid	0·0218	0·06	0·0478	0·16
Succinic acid	0·0102	0·03	0·3960	1·32
Residual carbohydrate	—	—	0·3200	—
	3·3750	6·163	2·2875	5·98
Original carbohydrate	2·3230	—	2·1460	—

Preparation of barium hexosemonophosphate.

The combined filtrates from the extraction of the crude barium hexosediphosphate were united and treated with a solution of basic lead acetate until precipitation was complete. The lead was removed by suspending the precipitated lead salts in water and treating with hydrogen sulphide. To the filtrate from the lead sulphide, hot baryta and an equal volume of alcohol were added to precipitate the crude barium hexosemonophosphate.

To purify the barium hexosemonophosphate it was extracted with 10 % alcohol, filtered, and reprecipitated with an equal volume of alcohol, washed frequently with absolute alcohol and dried *in vacuo*. On analysis of 1 g. of this product, which dissolves quite easily in water, giving a clear solution, its glucose equivalent was found to be 0·2720 g. There was no trace of inorganic phosphate and the combined phosphorus was 0·0709 g. (calculated 0·0785).

Decomposition of sodium hexosemonophosphate by B. coli.

This partially purified product was converted into sodium salt and subjected to the action of the *B. coli* under aerobic and anaerobic conditions in the way employed in the experiments with the hexosediphosphate. For the aerobic decomposition 9 g. of barium hexosemonophosphate were employed and for the anaerobic decomposition 6 g.

Table II.

	Aerobic decomposition. Weight (g.) per 1 g. of Ba salt used	Anaerobic decomposition. Weight (g.) per 1 g. of Ba salt used
Carbon dioxide	0·184	0·117
Alcohol	0·028	0·052
Acetic acid	0·152	0·085
Formic acid	0·005	0·009
Lactic acid	0·004	0·056
Succinic acid	0·002	0·038
Residual carbohydrate ...	0·019	0·024

In each case there was some residual carbohydrate unfermented and a small amount of phosphorus still in organic combination.

From the above tables it appears that both with the mono- and the di-phosphates oxygenation results in a marked decrease in the formation of the acids as well as of the alcohol, and a corresponding increase in carbon dioxide.

The high acetic acid content of the products of aerobic fermentation of sodium hexosemonophosphate would probably have been markedly diminished and the carbon dioxide formed increased if the oxygen supply had been more rapid. Acetic acid may possibly be one of the last stages in the decomposition of hexose by *B. coli*. In this regard the results are not similar to those found by Grey for the action of *B. coli* on glucose, who states that in this case the effect of introducing oxygen in the fermentation is to increase the lactic, acetic and succinic acids and to diminish the hydrogen, carbon dioxide and formic acid but to leave the alcohol unchanged. On the other hand the decomposition under anaerobic conditions is of the same character as that of the free sugars.

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L. A NOTE ON THE PRECIPITATION OF THE ANTISCORBUTIC FACTOR FROM LEMON JUICE.

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(*Received February 28th, 1927.*)

The method of fractionation of the antiscorbutic factor introduced by the author [1924, 1925, 1] is based on the facts that the active principle is precipitated by basic lead acetate and that inactive impurities after the removal of the acids from the juice are eliminated by precipitation with neutral lead acetate and alcohol. The main objection to this procedure is that the addition of basic lead acetate in excess brings the reaction of the resulting solution to the alkaline side of neutrality and thus a certain risk of inactivation is incurred. Evidently the stage at which the vitamin is precipitated by the lead acetate is controlled by the hydrogen-ion concentration of the medium [Zilva, 1925, 2], and it is now found that the major part of the activity is in the fraction precipitated at about p_H 7. This is seen from the following experiment. Lemon juice was neutralised with calcium carbonate and allowed to stand for about an hour, after which time it was filtered. An excess of neutral lead acetate was then added to the filtrate, and the reaction adjusted by the addition of dilute ammonia to p_H 7 (phenol red). The precipitate was finally centrifuged and decomposed in the usual way. This preparation was tested out on guinea-pigs (250–300 g.) by the daily administration of freshly prepared doses equivalent to 1·5 cc., 3 cc. and 5 cc. of the original lemon juice, three animals being used for each dose. On the lowest dose only one animal survived the test term of 60 days, showing scorbutic signs at the autopsy after being chloroformed; of the other two animals one died after 56 days and the other after 52 days. The guinea-pigs on the 3 cc. and 5 cc. doses were chloroformed after 60 days, and only in the former group were *very* slight signs of scurvy found at the *post-mortem* examination. It is, therefore, seen that the best part of the active principle was precipitated by the lead acetate at p_H 7. Traces of the antiscorbutic factor could be demonstrated in the filtrate by testing the precipitate obtained on raising the p_H of the solution to 7·5.

Further investigation has shown that most of the antiscorbutic factor is brought down by lead acetate within the p_H range of 5·4–7·2. Decitrated lemon juice as prepared above was treated with an excess of neutral lead acetate. After centrifuging the precipitate, the p_H of the supernatant solution

(5·4) was raised by the cautious addition of dilute ammonia to p_H 7·2. A further precipitate much smaller in bulk was formed, which, on being decomposed, was tested for its activity in the same doses and on the same number of animals as above. The activity was found to be of the same order.

As will be seen from the following table, in spite of the undiminished antiscorbutic activity there is a great reduction in the total solids and in the sugar-content of this preparation. This fraction which is quickly and conveniently prepared can therefore be utilised as a suitable starting-point for further purification.

Table I.

Fraction composed of the total precipitate at p_H 7

No.	Dry matter (%)	Reducing power as glucose (%)
1	1·610	0·580
2	2·432	0·930
3	2·136	0·800

Corresponding fraction obtained by precipitation between p_H 5·4–7

No.	Dry matter (%)	Reducing power as glucose (%)
1	0·1278	0·041
2	0·1800	0·070
3	0·199	0·053

The author wishes to express his thanks to the Medical Research Council for a whole-time grant.

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LI. THE PROTEIN ERROR IN ESTIMATING p_H WITH NEUTRAL RED AND PHENOL RED.

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(*Received March 1st, 1927.*)

Sørensen [1910, 1912] first systematically studied the protein error of the indicators then available, including neutral red. The solutions of protein contained sodium chloride and a phosphate buffer, and the p_H was estimated electrically and colorimetrically. He found that 2 % gelatin had no colorimetric error, 2 % peptone gave readings about 0·13 p_H too low and 2 % crude egg-white 0·6 to 0·7 too low.

Homer [1917], using the undiluted sera of horses immunised against diphtheria containing 4 to 8 % of proteins, found that the colorimetric p_H with neutral red was in the majority of cases 0·02 to 0·11 too low, but that occasionally it was as much as 0·2 too high. Phenol red under the same conditions gave colorimetric results which were always 0·84 to 0·92 too low, the error being greater with the more alkaline sera. Atkin (private communication), using horse-serum diluted five times with 0·9 % saline, found that the colorimetric error with phenol red was 0·17 at p_H 6·74 and 0·41 at p_H 8·33. Cullen [1922] found that human plasma had to be diluted twenty times before the colorimetric p_H, as estimated with phenol red, agreed with the electrometric determination. A one-in-three dilution of the plasma determined colorimetrically gave 0·4 p_H lower than the electrical measurement.

In the course of some experiments connected with the concentration of horse-sera containing antitoxin, we had occasion to examine the error of the colorimetric determination of the p_H of a strong solution of horse-pseudoglobulin when phenol red and neutral red were used as indicators. The results obtained were so different from those which we had previously observed when using these indicators for the colorimetric determination of the p_H of whole serum that they suggested that pseudoglobulin and albumin behaved differently. We accordingly carried out a parallel series of observations with serum-albumin of the horse which had been six times recrystallised. We found that with both these indicators the error caused in the colorimetric determination of p_H by pseudoglobulin was in the opposite direction to that caused by serum-albumin. Serum-albumin diminished the colour of the indicators; pseudoglobulin increased it. The divergence between colorimetric

and electrometric is, as might be anticipated, in all cases opposite with neutral red to that with phenol red.

These results led us to examine also the effect of egg-white and recrystallised egg-albumin.

Observations on neutral red.

The p_H of a solution of pseudoglobulin, twice fractionated with $(NH_4)_2SO_4$, containing 16·5 % protein, was determined electrically. The solution was not entirely free from euglobulin. It was titrated electrometrically to the isoelectric point. From the $N/10$ acid required the amount of base in the globulin solution was calculated. Some $M/45$ phosphate having the same p_H electrically

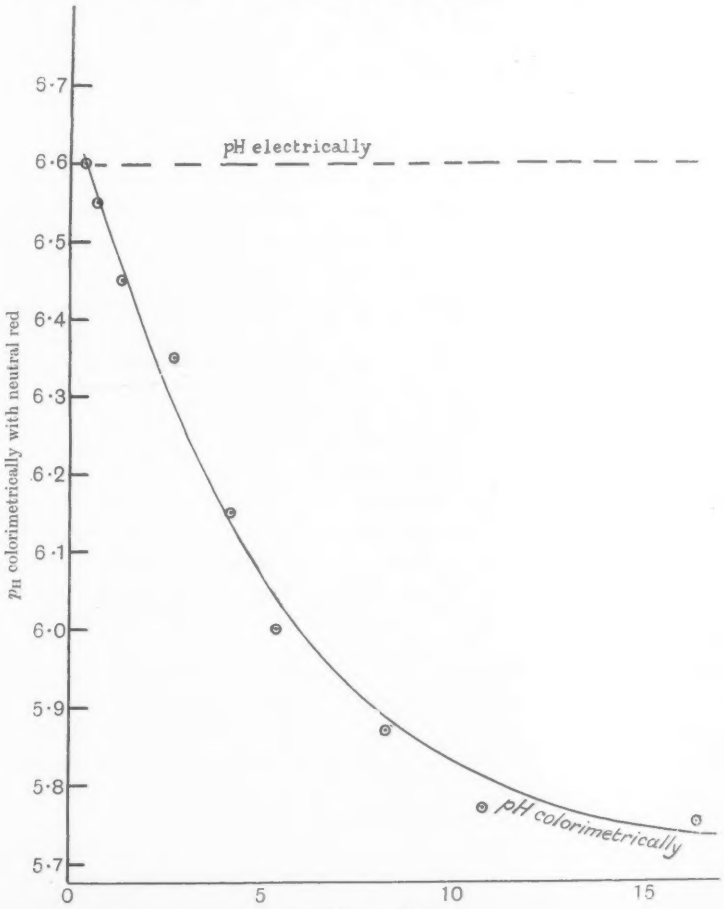

Fig. 1. Globulin error of neutral red.

as the globulin solution was prepared. This contained the same concentration of Na as the globulin solution. Standard phosphate tubes at intervals of 0·1 p_H containing neutral red as indicator were used for the colorimetric determinations.

Various dilutions of the globulin solution were made by adding the phosphate solution. The results are shown in Table I and graphically in Fig. 1.

Table I. *The pseudoglobulin error of neutral red.*

Globulin %	p_H colorimetrically	Globulin %	p_H colorimetrically
16·35	5·75	2·7	6·35
10·8	5·77	1·35	6·45
8·17	5·87	0·67	6·55
5·4	6·0	0·33	6·60
4·09	6·15	0·17	6·60
		0·0	6·60

A similar experiment was carried out with recrystallised serum-albumin. In this case the strongest solution contained 3 % albumin, $M/60$ phosphate and 0·85 % sodium chloride. It was diluted with $M/60$ phosphate which contained the same concentration of sodium and had the same p_H as that found electrically for the albumin solution. The results are shown in Table II and Fig. 2. The effect is in the opposite direction to that with globulin. The serum error of neutral red will therefore depend on the proportion of albumin to globulin in any particular sample.

Table II. *The serum-albumin error of neutral red.*

Albumin %	p_H colorimetrically	Albumin %	p_H colorimetrically
3·0	6·93	0·095	6·45
1·5	6·86	0·047	6·35
0·75	6·75	0·23	6·35
0·38	6·65	0·0	6·35
0·19	6·55		

Ledingham [1907] found that in horses immunised against diphtheria the globulin content of the serum increased. The serum of one horse at the beginning of treatment contained 5 % globulin and 2·7 % albumin. This serum would, from our curves, have a colorimetric error of 0·58 less 0·60 so that the p_H would be 0·02 too low. Six weeks later this horse's serum contained 8 % globulin and 2 % albumin and the colorimetric error would now have been 0·51 less 0·74 or 0·23 too low.

We next tested the effect of purified egg-albumin and found to our surprise that a 7 % solution of six times recrystallised egg-albumin showed no colorimetric error. Crude egg-white, however, 20 % by volume, gave a p_H 0·52 too low. Sørensen [1910] with 2 % egg-white found the p_H colorimetrically 0·6 to 0·7 too low.

Observations with phenol red.

In these experiments the protein was always made up with $M/60$ phosphate and enough sodium chloride added to bring the molecular concentration of sodium to approximately $M/7$, for we have shown [Lepper and Martin, 1926] that the dissociation of phenol red is sensitive to cations and in order to get identical results electrically and colorimetrically when using $M/15$ phosphate

solutions as standards it is necessary to have the molecular concentration of the sodium in the solution to be tested approximately the same as in the standards. The dilutions were made with $M/60$ phosphate containing sodium chloride adjusted colorimetrically to the p_H required.

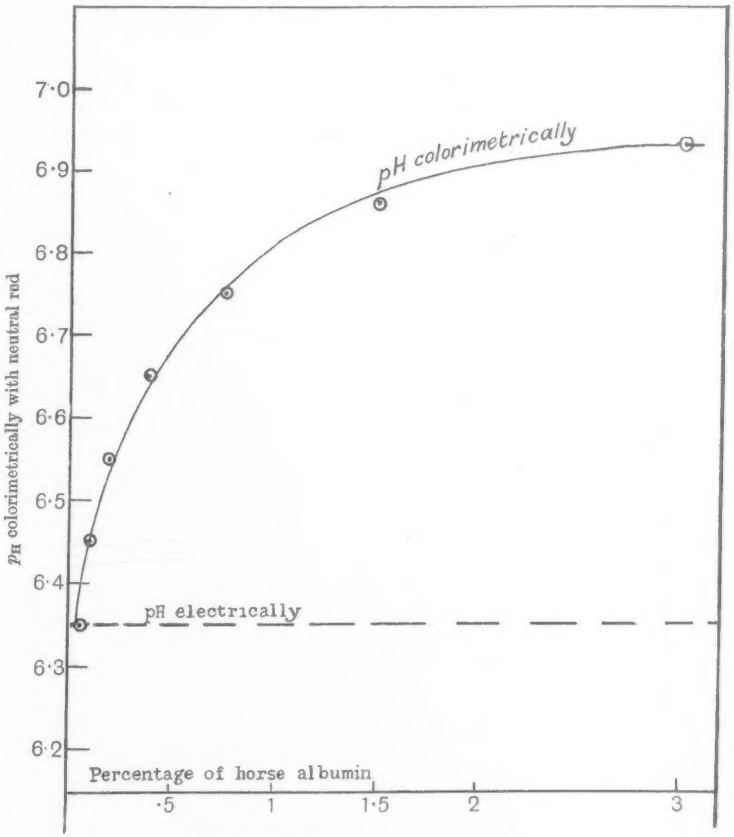

Fig. 2. The albumin error of neutral red.

The results with globulin are shown in Table III. Pseudoglobulin causes a slight rise in the apparent p_H which is more easily detected at the alkaline end of the indicator range. 2 % has no effect.

Table III. *The pseudoglobulin error of phenol red.*

Globulin %	p_H colorimetrically	Globulin %	p_H colorimetrically
11·0	8·05	12	7·42
7·0	8·02	8	7·41
3·5	7·95	4	7·40
1·75	7·93	2	7·38
0·0	7·93	0	7·38

Table IV. *The serum-albumin error of phenol red.*

Albumin %	p_H colorimetrically	Albumin %	p_H colorimetrically
3	7·69	0·125	7·99
2	7·73	0·0625	8·0
1	7·80	0·031	8·02
0·5	7·86	0·0	8·02
0·25	7·94		

Albumin, however, produces a marked decrease in the apparent p_H as shown in Table IV and Fig. 3; this was slightly greater the higher the p_H. With serum the albumin error will, therefore, always predominate. Cullen [1922] with human serum and a bicarbonate buffer found a higher colorimetric error than we have done with serum-albumin of the horse.

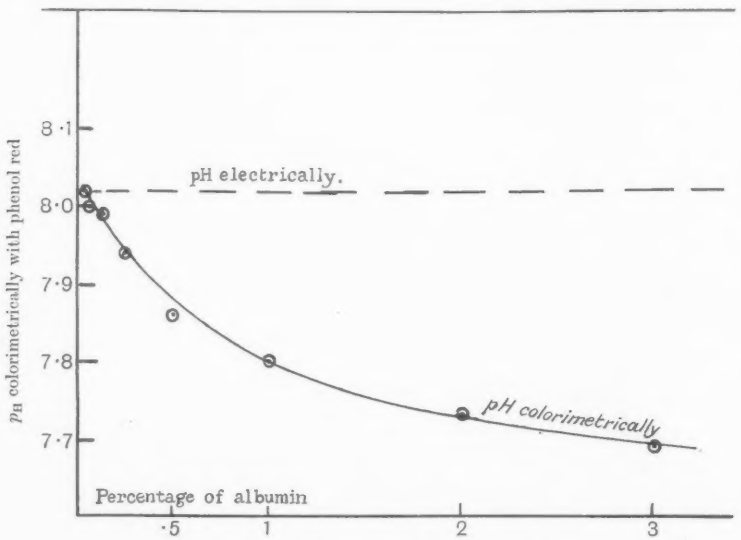

Fig. 3. The albumin error of phenol red.

The presence of pure egg-albumin again caused no error but crude egg-white, 20 %, decreased the p_H by 0·1.

Discussion.

The results obtained show that the protein error of an indicator must be determined for each protein. The effect of serum, for instance, is a summation of the opposed effects of the albumin and globulins. These may almost balance each other, as happens in the case of neutral red, or one error may predominate as in the case of phenol red. Proteins belonging to the group of albumins but derived from different sources do not react in the same way with a particular indicator; with neutral red the error is considerable with

horse-albumin but negligible with egg-albumin. The experiments do not throw any light on the way in which proteins interfere with the colorimetric determination of p_H. With the indicators used, there was no precipitation of the dye or alteration in colour such as occurs, for instance, with bromo-*o*-cresolsulphonephthalein which gives a blue colour in the presence of proteins impossible to match with the standards. The diminution of colour of both types of indicator in the presence of albumins might be due to diminished dissociation or adsorption on the colloid particles, but an increase in the presence of globulins requires the assumption of complexes between the dye and protein possessing a higher dissociation coefficient.

SUMMARY.

The protein errors with neutral red and phenol red for pseudoglobulin and serum-albumin of the horse and for egg-albumin and egg-white in varying concentrations, have been determined.

With both these indicators the colorimetric error due to the presence of this pseudoglobulin is in the opposite direction to that caused by serum-albumin. In either case the errors with neutral red are in the opposite sense to those with phenol red.

Pure egg-albumin up to 7 % does not cause any colorimetric error with either neutral red or phenol red.

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LII. ON THE ABSORPTION OF VITAMIN D FROM THE SKIN.

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An animal can either obtain the necessary supply of vitamin D for skeletal growth from its food or it can make it for itself from some precursor, if its surface is sufficiently exposed to sunlight or ultra-violet rays.

The principle which is activated to form vitamin D has hitherto been found associated with cholesterol, but from observations of Rosenheim and Webster [1926; 1927, 1, 2] and Heilbron, Kamm and Morton [1927] the precursor of vitamin D is not ordinary cholesterol, although it clings to it during successive recrystallisations. Rosenheim and Webster [1927, 2] adduce good reasons for supposing that it is ergosterol. They find that ergosterol is 2000 times as rich in the provitamin as brain cholesterol. Ergosterol unlike ordinary cholesterol has three double bonds and has three absorption bands in the ultra-violet, the strongest being at a wave length of 280 $\mu\mu$, accompanied by two others of less intensity, at wave lengths of about 270 $\mu\mu$ and 295 $\mu\mu$. Hess and Weinstock [1925] have shown that "cholesterol" irradiated by ultra-violet rays exerts its anti-rachitic action when injected subcutaneously as well as when taken by the mouth. They also found [Hess, 1925; Hess and Weinstock, 1925] that pieces of irradiated skin from infants and calves exercised an anti-rachitic action when eaten by rats. Hess, Weinstock and Helman [1925] showed that lanolin contains the precursor of vitamin D and suggested that it is by the activation of the cholesterol in the layers of the epidermis and its subsequent absorption that animals are protected from rickets. Yet another possibility is that the precursor is activated whilst coursing through the superficial capillaries of the skin, but for this to occur, sufficient radiant energy of the required wave length must penetrate the 0·1 to 0·2 mm. of the epidermis covering the capillaries. The possession by ergosterol of a strong absorption band about the wave length of 280 $\mu\mu$ [Rosenheim and Webster, 1926] indicates that light of this wave length would be particularly effective in activating it. The epidermis, however, is very opaque to this wave length. One of us (N. S. L.) has not succeeded in obtaining any indication of the 280 $\mu\mu$ line in a spectrogram of a quartz mercury vapour lamp, distant 2 feet, after 3 hours' exposure, through 0·1 mm. of epidermis when using an Ilford Process plate (H. and D. = 50).

Radiations of wave length 280 $\mu\mu$ are not present to a significant extent in sunlight which has passed through the upper atmosphere [Cornu, 1878, 1879, 1880, 1890; Miethe and Lehman, 1909; Dember, 1912; Wiegand, 1913] so that the precursor of vitamin D must be capable of being activated, if more slowly, by ultra-violet light of greater wave length impinging on the body. For ultra-violet light of slightly greater wave length, the epidermis is much less opaque. Calculating from Hasselbalch's [1911] observations, 1/56 of the incident light of a wave length of 297 $\mu\mu$ penetrates 0·1 mm. and 1/3000 penetrates 0·2 mm. of epidermis. At present we are ignorant of the amount of radiant energy required to activate sufficient of the precursor to provide an adequate supply of vitamin D for an animal, but it would appear to be very small, when of the appropriate wave length; for 1½ minutes' daily exposure to a mercury vapour quartz lamp is sufficient to prevent rickets in an albino rat, for the most part covered with fur, and fed on a rickets-producing diet [Hess, 1922]. It was observed by Chick and co-workers [1923] that rickets in a child was healed when the hand and fore-arm only were exposed to ultra-violet irradiation. Hess and Unger [1921] made a similar observation, but in this case the exposure was to the rays of the sun.

The accompanying experiments were designed to test whether irradiated cholesterol, applied to the external surface, could exercise its anti-rachitic action through the skin.

Technique.

Two methods were used for testing the potency of the various anti-rachitic measures tried. For the first method, young rats were used, which at about 50 g. weight had been placed upon a diet deficient in both fat-soluble vitamins; the method, which has been described before [Hume and Smith, 1926], takes advantage of the fact that the animal's reserve of vitamin D is exhausted before that of vitamin A. After the rats had been on this diet for 4 weeks the treatment to be tested was applied, and the degree to which growth was restored was taken as a measure of the anti-rachitic potency of the treatment.

For the second method, young rabbits, between 300 and 500 g. weight, were used and they were fed on the diet No. 3143 of McCollum [1921], which is low in phosphorus. They also received on week days 5 cc. of orange juice, given with a pipette; it could not be mixed with the diet, as this was given dry. On this diet rabbits develop gross lesions of rickets, unless anti-rachitic treatment of some sort is applied. In the first experiment the rabbits were kept on the diet for 8 weeks and in the second for 5 weeks. At the end of the experiment a femur from each rabbit was ashed, after extraction with ether and alcohol. The histology of the rib junctions was also studied.

The part of the body chosen, as most inaccessible to the animal itself, for the application of irradiated cholesterol was the middle of the chest in rats and the back of the neck in rabbits. The skin was kept as clear of hair as

possible by depilation and cutting of the hair. Depilation was at first carried out with barium sulphide, but in the experiment with rats and in the first experiment with rabbits, it caused slight soreness, so the experiment with rabbits was repeated using toilet "Veet," in which the barium sulphide is diluted with a cream; no soreness then resulted.

About 0·2 g. of cholesterol was dissolved in ether and evaporated on a glass slide in order to provide a thin layer. This was irradiated for 10 minutes, at a distance of 16 inches from a mercury vapour quartz lamp (Ulviarc), freshly for every application, and the application was made three times a week. The cholesterol was gently applied to the skin with the point of a finger, over an area of about one square inch, and a little hardened cotton-seed oil was afterwards applied to make the cholesterol adhere. The animals were dressed in chamois-leather jackets, fastening down the back, which prevented them from gaining access in any way to the treated patch of skin and each animal was kept in a separate cage. It was found extremely difficult to be perfectly certain that no particle of irradiated cholesterol should accidentally reach the animals' mouths, and the experiment was performed three times, each time with added precautions, but each time with the same result.

Table I. *Growth of rats on a diet deficient in fat-soluble vitamins; irradiated cholesterol applied to the skin from 27th–47th day.*

Treatment	No. of rat	Sex	No. of litter	Weight on 27th day g.	Weight on 47th day g.	Total increase g.
Control	L 1	♀	310	52	60	8
	L 3	♂	.	68	78	10
	L 4	♂	„	71	84	13
Irradiated cholesterol applied to skin	L 2	♀	310	51	73	22
	L 5	♂	„	67	86	19
	L 6	♂	„	66	81	15

Exp. 1. In the first experiment, six white rats of the Wistar Institute breed, all belonging to the same litter, were used. They all received the diet, deficient in fat-soluble vitamins, already referred to. After 27 days on the diet, irradiated cholesterol was applied to a depilated patch on the chests of three of the rats, three times a week for 20 days. The remaining three were left untreated as controls. Table I shows the growth of the two sets of animals, over the twenty days of treatment. The three treated animals show growth which is about double that of the untreated animals and it would therefore appear that by inunction the irradiated cholesterol has been able to exercise a definite effect. It was however felt that by using a larger animal it would be considerably easier to take the necessary precautions against the entry of the anti-rachitic agent, by any other channel than through the intact skin. It was therefore decided to use rabbits.

Exp. 2. Five rabbits, all stated to belong to one litter, were used. They all received the diet, McCollum 3143. Two received no further treatment; irradiated cholesterol was rubbed into the shoulders of two 3 times a week,

and one was irradiated directly, with a mercury vapour quartz lamp for 10 minutes daily, at a distance of 46 cm. Treatment lasted for 8 weeks. Table II A, which gives the results, shows that both histologically and in their percentage ash the bones of the animals which had had cholesterol applied to their skins approached those of the animal irradiated directly, while the controls showed rickets histologically and a considerably lower percentage of ash.

The two animals which received cholesterol, were, however, at the end of the experiment, slightly sore and it was also felt that in order to imitate any natural absorption which might take place, the irradiated cholesterol should have been gently stroked on the skin, and not rubbed in as had so far been done. The next experiment was also planned to include rabbits irradiated directly, over a small area of the body only.

Exp. 3. In Exp. 3, seven rabbits, stated to belong to two litters, were used. Depilation was carried out by means of "Veet." The diet was the same as in the last experiment, but treatment was only continued for 5 weeks. Two of the rabbits were used as untreated controls. On two, a patch was depilated at the back of the neck, and three times a week irradiated cholesterol was very gently stroked on to the skin and a little hardened cotton-seed oil was lightly smeared over it. The application was only made to a small area of about one square inch and it was felt during the experiment that, if the treatment in so slight a form could produce an effect, it must be very potent. From Table II B it will be seen that a very definite effect was produced and though the results obtained are not quite as good as in the case of animals irradiated directly, yet they are very much better than the results yielded by the controls, one of which showed an extraordinarily low ash in the bone.

Table II. *Histological report on the rib junctions and percentage ash in the extracted bone of rabbits, fed on diet* 3143 *of McCollum, and treated with irradiated cholesterol applied to the skin, or with direct irradiation of whole or part of the body.*

Treatment	No. of rabbit	Sex	No. of litter	% ash in extracted bone	Histological report
A. Control	2	♀	1	46·8	Severe rickets
	5	♀	,,	48·1	,,
Irradiated cholesterol applied to skin	3	♀	,,	56·3	No rickets
	6	♂	,,	55·7	,,
Irradiated directly ...	4	♀	,,	58·0	,,
B. Control	8	♂	2	46·2	Moderate rickets
	13	♀	3	37·1	Very severe rickets
Irradiated cholesterol applied to skin	7	♀	2	52·9	No rickets
	10	♂	3	52·5	Slight rickets
Irradiated through hole 2·5 × 3·5 cm.	9	♂	2	56·4	—
	11	♂	3	55·6	—
	12	♂	3	57·0	—

The three remaining rabbits were irradiated directly through a small hole in a black cloth. The cloth was of black sateen doubled. Penetration of light

through the cloth was tested by means of a spectrograph; the spectrogram showed no penetration by ultra-violet rays, after half an hour's exposure to a mercury vapour arc. The hole in the cloth measured 2·5 by 3·5 cm. The rabbit was rolled in the black cloth and a depilated area on its back was exposed directly through the hole, at a distance of 50 cm. to a mercury vapour quartz lamp. The exposure lasted 10 minutes and took place three times a week. Approximately, but not quite, the same area of skin was irradiated at each exposure. With one rabbit, it was attempted to cleanse the depilated area with ether and free it from cholesterol, but this was found to be quite impossible and the rabbit, No. 12, is therefore included with Nos. 9 and 11, in the group of those irradiated directly through a hole.

All the rabbits in Exp. 3 were killed when they were slightly younger than the rabbits in Exp. 2, so that a slightly lower range of values for the percentage ash might be expected in Exp. 3. Allowing for this it is apparent that calcification in an animal, of which only a small area has been irradiated periodically for 10 minutes, is only slightly inferior to that of an animal whose whole body has been irradiated for the same time.

Conclusions.

1. Vitamin D in irradiated cholesterol can be absorbed from a small area of undamaged skin in sufficient amount to supply the needs of the animal.

2. The suggestion of Hess that activation of lanolin by sunlight and its subsequent absorption through the skin is a possible source of this vitamin is supported by experiment.

3. Rickets was prevented in rabbits fed upon a rickets-producing diet, and almost normal calcification of the bones produced, when an area of skin 2·5 × 3·5 cm. was irradiated for ten minutes three times a week.

The writers' thanks are due to Prof. C. J. Martin for kind help and advice, to the Lister Institute for its hospitality, and to the Medical Research Council for a whole time and a half time grant for salaries of two of the workers.

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LIII. A SEARCH FOR VITAMIN D IN THE DIATOM *NITZSCHIA CLOSTERIUM* (W. SM.).

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JAMESON, DRUMMOND and COWARD [1922] showed that the marine diatom *Nitzschia closterium* (W. Sm.) *forma minutissima*, grown in sterilised sea-water with the addition of Miquel's nutrient solutions, can synthesise large amounts of fat-soluble vitamin, and they came to the conclusion that the ultimate though indirect source of the fat-soluble vitamin was this and other marine unicellular organisms.

In view of the recent differentiation of the fat-soluble vitamins, it was suggested to me by Dr H. Chick that it would be interesting to see whether *Nitzschia closterium* also contained vitamin D.

It has been found that the green land plants examined up to the present contain very little, if any, vitamin D [Goldblatt and Zilva, 1923; McClendon and Shuck, 1923; Zucker and Barnett, 1923; Hess and Weinstock, 1924; Bethke, Kennard and Kik, 1925; Chick and Roscoe, 1926; Boas, 1926; Roscoe, 1927] and it was thought that it would be of interest to ascertain whether the same held for marine unicellular organisms containing chlorophyll. There is good reason to believe that these are the ultimate source, via plankton, copepods and small fish, whence deep-sea fish, such as the cod, indirectly obtain their supplies of vitamin A; but it is not known whether the store of vitamin D, found in the liver oils of the codfish, is obtained in this way.

TECHNIQUE.

The experiments described below were carried out on four different litters of rats. The basal diet given was McCollum's No. 3143 low phosphorus rickets-producing diet [1921].

A culture of *Nitzschia closterium* was kindly supplied to me by Professor Drummond, of University College, London, and from this further cultures were grown as described by Jameson, Drummond and Coward [1922]. At first the cultures were kept in Erlenmeyer flasks which were placed in a window facing north. They were never exposed to direct sunlight and all the light which reached them had to pass through a pane of plate-glass and the glass

wall of the flask. Very little ultra-violet light of wave-length less than 340 $\mu\mu$ succeeded in reaching the *Nitzschia* in the flasks. It was therefore decided to try to grow the diatom under conditions more comparable to those to which it is exposed when floating in the superficial layers of the sea, and to grow it in shallow glass dishes on a flat roof to catch all the sunlight available, shielding it from bacterial contamination by a sheet of "windowlite."

A spectrogram of the windowlite used was kindly made for me by Dr Lucas. This showed that windowlite has a fair transparency for ultra-violet light of wave-length longer than 290 $\mu\mu$ or nearly to the extreme range of the sun's spectrum. A padding of sterilised cotton-wool was introduced between the edge of the loose-fitting lid and the rim of the glass vessel in order to preserve the culture from contamination. The *Nitzschia* grew well under these conditions throughout the months of September and October.

Each litter of young rats received the dose of *Nitzschia* after somewhat different treatment. At first, the culture was filtered to dryness on a layer of starch in Gooch crucibles or Büchner funnels, and then further dried for two hours before administration, being mixed with starch in the proportions of 1 to 3. Later the drying at 37° was omitted, and in the final experiment the *Nitzschia* was given without any starch. In this last case, the diatom was filtered freshly every day from the glass dishes on the roof.

The amount of water lost, when *Nitzschia* was collected on a Büchner funnel, and dried at 100° to constant weight, was from 70–75%. The actual weight of dried material administered would therefore be one-quarter of that of the fresh diatom. The doses specified below refer to the fresh weight of the diatom, after suction on the Büchner funnel.

The *Nitzschia* was always given before the basal diet. It was eaten readily after the first few days. Each batch of the diatom was examined microscopically before filtering to make sure both that the culture was free from other diatoms and also that the diatom was alive and in a healthy condition.

To test the condition of the skeletons of the rats the junctions of the 6th and 7th ribs on the right side were examined histologically, and chemical analyses were made on the leg bones (femora, tibiae and fibulae). The methods used are described in full in the paper by Chick, Korenchevsky and Roscoe [1926].

Results.

Exp. 1. The *Nitzschia* was grown in Erlenmeyer flasks in diffused light passing through plate-glass windows. The culture was filtered on starch, dried at 37° and administered with starch as described above.

A litter of five black-and-white rats was put on experiment on June 26th, 1926. The experiment lasted four weeks. Two of the rats, one male and one female, were used as controls; two others, one male and one female, received 0·1 g. (fresh weight) of *Nitzschia* in addition to the basal diet. One rat received 60 mg. of cod-liver oil.

The histological and chemical analyses of their bones, together with the initial weight and growth of the rats, will be found in the table.

Experiment	No. of rat	Sex	Dose of *Nitzschia* g. fresh weight	Initial weight g.	Increase g.	Duration of exp. (days)	Degree of rickets from histology of rib junctions	Ca % in leg-bones on fat-extracted dry weight
I	7	♂	Control	45	16	26	+ + + +	9·7
	5	♀	Control	38	27	26	+ + + +	9·4
	10	♂	0·1	41	30	26	+ + + +	7·9
	4	♀	0·1	37	23	26	+ + + +	7·3
	3	♂	60 mg. C.L.O.	40	25	26		13·1
II	16	♂	Control	46	17	19	+ + +	8·0
	17	♀	Control	42	11	20	+ +	8·4
	12	♂	0·2	42	7	19	+ +	7·6
	13	♀	0·2	41	14	20	+ +	8·3
	14	♂	0·1	41	12	19	+ + +	7·1
	15	♀	0·1	39	20	20	+ + + +	8·0
III	18	♂	Control 16 days	41	12	16	+ +	9·8
	21	♂	0·21 after 16 days	41	18	23	+ + + +	8·3
	22	♀	Control 18 days	42	10	18	+ + +	10·0
	25	♀	0·21 after 18 days	41	18	25	+ + + +	9·3
	20	♂	Control 21 days	44	12	21	+ + +	9·4
	23	♂	0·21 after 21 days	41	25	28	+ + + +	9·5
	24	♀	Control 23 days	39	19	23	+ + +	8·9
	19	♀	0·21 after 23 days	39	12	30	+ +	10·9
IV	26	♀	Control	41	35	21	+	13·0
	27	♀	Control	50	24	21	+ +	10·6
	29	♀	An average of ·378 filtered fresh daily	39	34	21	+ + +	10·3
	30	♀		39	28	21	+ + +	10·1

It will be seen that all the rats except No. 3, which was receiving cod-liver oil, were intensely rachitic; it was impossible to find any significant difference between them histologically. In the chemical analysis it will be seen that, disregarding the animal receiving cod-liver oil (No. 3), the bones of the control animals (Nos. 7 and 5) had a higher percentage of calcium than those of the animals receiving the supplementary rations of *Nitzschia*.

Exp. 2. The *Nitzschia* given had been grown in Erlenmeyer flasks for six weeks in the same manner as in Exp. 1. At the end of this period it was put into the shallow glass dishes and exposed on the roof, as described at the beginning of this paper. The diatom was filtered off on to starch, dried and administered as in Exp. 1.

A litter of six white rats was used. The experiment was started on Sept. 17th, 1926. Two of the rats, a male and a female, were used as controls. These received only the basal diet. Two were given 0·2 g. of *Nitzschia* and two 0·1 g. in addition to their basal ration.

The experiment lasted 19–20 days. There was no striking difference in the calcium content of the bones of the animals, though in each case the bones of the control rats had higher calcium-content than those of the rats of the same sex receiving *Nitzschia*. The histological examination showed little difference between the various animals. They were all rachitic.

Exp. 3. The *Nitzschia* was propagated at first indoors and transferred to the roof after three weeks. It remained on the roof for a week or more

before being filtered off. It was given as before, mixed with starch in the proportion of 1 in 4.

A litter of eight black-and-white rats was used. The experiment was commenced on Sept. 27th, 1926. Four rats were taken as controls and were killed after 16, 18, 21 and 23 days respectively. Four other rats of corresponding sex received doses of *Nitzschia* for one week after being on basal diet alone for 16, 18, 21 and 23 days respectively. These rats were then killed. There was very little difference in the calcium content of the bones of two groups of animals. Two controls showed a higher calcium content in their bones than the corresponding animals fed with *Nitzschia* for a further week, and two other controls showed a lower content. Histologically, also, there was little to choose. They all had rickets in a marked degree.

Exp. 4. The culture of *Nitzschia* was started indoors for a short time and subsequently grown on the roof and filtered fresh daily for administration to the rats.

A litter of 4 black-and-white female rats was put on experiment on October 7th, 1926. Two of these rats were used as controls and two received *Nitzschia* filtered fresh daily. Their daily dose averaged 0·40 g. (fresh weight) during the first, 0·46 g. during the second and 0·27 g. during the third week.

Chemical analysis of the bones showed that the calcium content of the bones of the control animals was not lower than that of the rats receiving freshly filtered *Nitzschia*.

Discussion.

From a paper by Chick and Roscoe [1926], it appears that the anti-rachitic potency of cow's milk is very little increased by feeding the animal on green pasture food while the cow itself is kept in a dark stall. It is only when the cow is itself exposed to the rays of the sun that the anti-rachitic value of its milk increases appreciably. Cows fed on cod-liver oil in a dark stall, however, yield a milk of increased anti-rachitic potency [Golding, Soames and Zilva, 1926]. It seems as if it may be possible to trace a parallel in this respect between land and marine animals.

There are at present only two known sources from which the cod could obtain its supplies of vitamin D:

(1) The activation of the precursor of vitamin D by direct action of the sun's rays on the cod.

(2) The food it consumes.

It is difficult to suppose that the cod, a deep-sea fish, is ever exposed to enough sunshine to activate a precursor of vitamin D, so presumably it must obtain its supplies from the food which it consumes. The ultimate source of vitamin A in cod-liver oil has been traced by Zilva and Drummond [1922] to unicellular marine organisms. They say: "It is not directly from these organisms that the cod receives its vitamin but through several intermediaries such as the copepods and larval decapods and mollusca which are present in

plankton and which feed on these unicellular plants. These in turn are consumed by the capelan and other forms of food of the cod."

From the experiments on *Nitzschia closterium* described in this paper it does not appear probable that unicellular marine organisms are the ultimate source of vitamin D in cod-liver oil, and if this is correct there remain to be examined the plankton and the smaller fish, both of which might conceivably be exposed to sufficient radiant energy of the necessary wave-length for synthesis of vitamin D.

My thanks are due to Professor C. J. Martin, Dr H. Chick and Dr Zilva for kind criticism and help, to Professor Drummond for the culture of *Nitzschia*, to the Medical Research Council for a whole time grant, and to the Lister Institute for the hospitality of its laboratories.

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LIV. THE CARBOHYDRATE AND FAT METABOLISM OF YEAST.

IV. THE NATURE OF THE PHOSPHOLIPINS.

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After yeast has been incubated in a well-oxygenated solution of glucose, fructose or sucrose, its fat-content becomes very considerably increased. If the solution in which the yeast is incubated contains not only sugar but also a mixture of alkali phosphates, the total amount of lipoid (ether-soluble matter) which can be extracted from the yeast after its incubation is double that which can be extracted from the same amount of yeast incubated in a similar sugar solution from which phosphates are absent [Smedley-MacLean and Hoffert, 1923]. After incubation in the sugar-phosphate medium the phosphate-content of the yeast is also greatly increased, the increase depending on the concentration of the *sugar* in the medium. After yeast has been incubated in a solution containing only alkali phosphates, no increase in the phosphate-content of the yeast is observed [Smedley-MacLean and Hoffert, 1924].

This effect of the addition of phosphates is very striking and the inference seems permissible that the phosphates themselves play some part in the transformation of carbohydrate to fat. Evidence was adduced to show that the first stage of this change probably consists in the formation of a hexose-phosphate.

Again, in the complex lipin molecule phosphoric acid is known to occur in combination with fatty acids. The relation of the fatty acid to the lipin molecule is still unknown: the fatty acids may first be formed and then built up into the lipin molecule, or perhaps the whole change from carbohydrate to fatty acid may take place while the various groups are associated together in some complex molecule of which phosphoric acid forms an integral part.

Since the formation of fat from carbohydrate in yeast can be very largely influenced by altering the constituents of the medium in which the yeast is incubated, it seemed important to examine the nature of the yeast phospholipins, the proportion which these bear to the other constituents of the lipoid matter and the changes which take place when the composition of the medium is altered, especially by the addition of phosphates.

Previous work on yeast lipins.

As early as 1866, Hoppe-Seyler [1866] showed that an ether-soluble substance was present in yeast containing both nitrogen and phosphorus. Nägeli denied this but Hoppe-Seyler [1879] published a further paper in which he showed that of the substance extracted by ether from yeast the unsaponifiable matter constituted 24·14 % and at least another 14 % consisted of lipin. From this lipin he isolated both choline and glycerophosphoric acid.

Koch [1902] showed that the ratio of methyl groups to nitrogen atoms, instead of being 3 : 1 as it is in lecithin, in the yeast lipin was only 1·3 : 1, a ratio very much more nearly akin to that found in kephalin. Koch argued therefore that choline could not be the chief base of yeast lipin.

Sedlmayer [1903] found choline and isolated palmitic acid but failed to find oleic acid. A preliminary note published by Austin [1924] indicated the presence of both lecithin and kephalin in yeast, since both choline and hydroxyethylamine were detected, thus confirming the previous finding of Koch.

The fatty acids of yeast fat have been examined by a number of observers and oleic, linolic and palmitic acids have been identified with certainty and evidence has been adduced that lauric acid is probably present; small amounts of a higher melting acid, possibly arachidic, have also been found [Hinsberg and Roos, 1904; Neville, 1913; Smedley-MacLean and Thomas, 1920].

Method of experiment.

A series of experiments was first carried out in order to determine the changes which occur in the phosphorus-content of pressed yeast and in the proportion of its unsaponifiable matter after the yeast has been incubated in the sugar solution and in the sugar-phosphate medium respectively.

A known weight of yeast was incubated for 24 hours in a litre of 4 % sugar solution and a similar quantity of yeast in a litre of the same solution to which 3·962 g. NaH_2PO_4 and 0·286 g. K_2HPO_4 had been added. A rapid current of oxygen was passed through both liquids; it is essential that the rate of oxygen current should be as nearly as possible the same in both experiments. The yeast was then filtered off and analysed. The easiest method of extracting the fat is to boil the yeast for two hours with normal hydrochloric acid and to extract the fat from the residue. The lipin is however decomposed by this method and it is therefore necessary to separate the fat from the yeast by repeatedly extracting the dried yeast with alcohol. It was generally found necessary to make from ten to twelve extractions of the yeast, using in each case from fifteen to twenty times its own weight of alcohol, the final extraction being carried out with boiling alcohol. The yeast was first treated with about twenty times its weight of 96 % alcohol to remove most of the water, the diluted alcohol filtered off, concentrated under diminished pressure to a small volume and the residue extracted with light petroleum. The alcohol from

subsequent extractions was evaporated under diminished pressure and the residues taken up in light petroleum.

In some of these experiments we determined the percentage of phosphorus in the fat obtained from each successive extract; no distinct regularity was observed: on the whole the phosphorus-content of the fat was higher in the earlier fractions and there was certainly no indication that the phosphorised fat was more firmly bound or more difficult to extract from the cell than the fat or other lipoid constituents. Since lecithin and kephalin each contain about 4 % of phosphorus, the amount of phospholipin was calculated on this basis from the phosphorus-content of the fat.

The results are set out in the following table.

Table I.

No. of expt	Total lipoid by alcohol extraction g.	Total P in yeast g.	Total P in lipoid g.	% P in lipoid g.	% N calc. as protein	% unsap. matter
I. *Analysis of 15 grams of original pressed yeast.*						
833	0·1736	0·083	0·0023	1·32	1·42	—
835	0·1554	0·096	0·0020	1·29	1·83	—
837	0·1378	0·097	0·0003	0·22	1·58	—
839	0·1729	0·088	0·0018	1·06	1·72	—
841	0·1552	0·085	0·0019	1·23	1·69	—
845	0·1637	0·074	0·0019	1·14	1·69	31·1
847	0·1702	0·104	0·0021	1·21	1·53	35·4
Mean values	0·1613	0·090	0·0018	1·07	1·64	33·2
II. *Analysis of similar amount of yeast after incubation in 4 % glucose solution.*						
833	0·3320	0·098	0·0043	1·45	1·23	31·9
835	0·5031	0·106	0·0026	0·51	1·58	—
837	0·3508	0·098	0·0028	0·82	1·58	—
839	0·5002	0·090	0·0044	0·87	1·58	—
841	0·4080	0·091	0·0043	1·04	1·53	—
843	0·3740	0·079	0·0034	0·94	1·61	32·3
845	0·5455	0·093	0·0053	0·97	1·52	27·3
Mean values	0·4305	0·094	0·0039	0·94	1·52	30·5
III. *Analysis of similar amount of yeast after incubation in 4 % glucose-phosphate solution.*						
833	0·8235	0·134	0·0067	0·81	1·26	—
835	0·9192	0·146	0·0076	0·80	1·49	—
837	0·7630	0·154	0·0059	0·78	1·42	—
839	0·9172	0·144	0·0060	0·65	1·56	—
841	0·8254	0·143	0·0070	0·85	1·61	—
843	0·5770	0·120	0·0043	0·74	1·61	36·2
845	0·7794	0·127	0·0041	0·53	1·48	28·4
Mean values	0·8008	0·138	0·0058	0·74	1·49	32·3

From the figures given in the above table it follows that 100 g. of the original pressed yeast contain 1·08 g. of ether-soluble substance and 0·29 g. phospholipin, the latter being calculated from the phosphorus-content. The proportion of lipin in most of the samples examined was appreciably higher than this but one sample differed from the others in having an extremely low phosphorus-content and brought down the average amount.

After incubation in glucose solution alone, 100 g. of yeast contained 2·87 g. of total lipoid matter and 0·65 g. phospholipin. After incubation in the glucose-phosphate medium the ether-soluble substance constituted 5·34 % of the original weight of pressed yeast and contained 0·97 g. of phospholipin. The

total amount of ether-soluble substance had increased to five times its original amount and that of phospholipin to three times its original value. The variations in the percentage of lipin found in the incubated yeasts were considerably less than those in the original samples of pressed yeast taken for investigation.

The variations in the amount of unsaponifiable matter were not sufficient and the number of determinations made were too few for us to be able to attach any significance to them. In all cases approximately 30 % of the ether-soluble matter consisted of unsaponifiable substance. Since the total amount of the unsaponifiable matter increases in proportion to the amount of fat formed and is often five or six times that originally present, it must have been made by the yeast from carbohydrate, and the process is aided by the presence of phosphate in the medium.

Preparation of ether-soluble material for the isolation of lipins.

As shown above, the average content of lipin in 100 g. of pressed yeast is only 0·30 g.: this is however raised to approximately 1 g. if the yeast be first incubated in the well oxygenated glucose-phosphate medium. We determined therefore to increase the proportion of lipoid substances in the yeast before extracting it with alcohol, by incubating it in a suitable medium. The long and tedious process of extracting a reasonable amount of starting material was thus expedited and a considerable saving of alcohol effected.

In one experiment 3 kg. of pressed yeast, obtained from liquid brewery yeast, well washed with water, were added to 30 gallons of a 4 % solution of cane sugar containing 0·369 g. Na_2HPO_4 and 0·0286 g. KH_2PO_4 %, and for 28 hours a rapid current of air was blown through the solution. The yeast was allowed to settle overnight and in the morning the solution was decanted, the yeast filtered through a cloth, rubbed up with alcohol and again filtered by suction. The pressed-out yeast was ground with one-quarter of its weight of sand and extracted five or six times with alcohol. In this way from 3 kg. of yeast about 100 g. of extract were obtained containing roughly 20 g. of lipin. Determination of the amount of fat in a sample of the same yeast after it had been hydrolysed, showed that it contained nearly 180 g. of lipoid matter.

Separation of phospholipins.

The phospholipin fraction was separated by repeatedly precipitating with acetone the ether solution of the total lipoid material, and was obtained as a white amorphous substance. Subsequently a further separation into lecithin and kephalin fractions was made (see p. 379).

Throughout all work with lipins it is advisable to keep all flasks, cylinders, etc., thoroughly flushed with nitrogen so that oxidation may, as far as possible, be prevented.

The N : P ratio of the lipin was nearly 1 : 1; the nitrogenous impurity which gives so much trouble in preparing lecithin from animal tissues does not seem to be present to any large extent.

Table II. *Analysis of lipin from yeast.*

	Theoretical figure for dioleyl-lecithin	Mixed lipin		$CdCl_2$-lecithin fraction	Kephalin fraction
% P	3·81	3·59	3·585	3·096	3·02
% N	1·72	1·87	1·44	1·79	1·38
N : P	1 : 1	1·15 : 1	0·89 : 1	1·28 : 1	1·01 : 1

Products of hydrolysis of the lipin.

The lipin is conveniently hydrolysed by boiling with 10 % sulphuric acid for 6 to 8 hours under a reflux condenser in a slow stream of nitrogen.

(a) *Nature of bases.* The aqueous hydrolysis liquid, after extraction with ether to remove fatty acids, was analysed to determine the total nitrogen (by Kjeldahl's method) and the amino-nitrogen (by the micro-method of Van Slyke). The proportion of choline to amino-ethyl alcohol can thus be estimated and the lecithin-kephalin ratio calculated.

The lipin from pressed yeast contained a larger proportion of kephalin than lecithin, whereas in that from "incubated" yeast they were present in nearly equal amounts. Only a small amount of material was available after purification and it was therefore impossible to effect a complete separation of lecithin from kephalin.

The following table shows the total and amino-nitrogen in the hydrolysis liquids together with the percentage composition of the lipin.

Table III.

	Lipin from pressed yeast		Lipin from incubated yeast			
	Without separation		Without separation		"Kephalin"	"Lecithin"
	(a)	(b)	(c)	(d)	(e)	(f)
Total N ...	0·1273 g.	0·0679 g.	0·0708 g.	0·1113 g.	0·0302 g.	0·0811 g.
Amino-N ...	0·1123 g.	0·0494 g.	0·0346 g.	0·0451 g.	0·0187 g.	0·0264 g.
Calculated						
% lecithin ...	27·3	11·8	51·15	59·5	37·96	67·5
% kephalin	72·7	88·2	48·85	40·5	62·04	32·5

Before its separation, the pressed yeast has been standing for some days in the wort and a considerable amount of autolysis has taken place. On the other hand, after the yeast has been incubated in the sugar-phosphate solution, from 80 to 90 % of the fatty matter has been freshly formed and this has only been standing in contact with the medium for a comparatively short time. In this freshly formed lipin it is interesting that the proportion of lecithin is greater than in the lipin derived from the pressed yeast. MacLean [1915] has shown that the proportion of lecithin present in the lipins extracted from the tissues is greater if the tissue is procured in as fresh a condition as possible and dried as quickly as possible. There seems some reason therefore to believe that both in yeast and in animal tissues autolytic changes may occur by which lecithin is converted into kephalin.

On the other hand yeast, when supplied with carbohydrate and phosphate but with no nitrogenous matter, has to use the substances stored in its own cells for its nitrogen supply and the relative amount of the two lipins formed may conceivably be influenced by this factor.

An experiment was carried out in which nitrogen was added to the medium in the form of ammonium sulphate. Unfortunately for our purpose the addition to the medium of a nitrogenous substance resulted in an increase of the total weight of yeast and in a diminution of the total weight of fat present. In this case, protein is made in preference to fat so that we can only investigate the case of increased fat formation when the nitrogen is obtained from substances originally present in the yeast cell.

(b) *Nature of acids.* The fatty acids from the hydrolysed lipin were extracted with purified ether immediately after cooling, and amounted to 50–60 % of the original material; the iodine value of an aliquot portion of the extract showed 60–70 % of the acids to be unsaturated.

The mixture was separated into the liquid unsaturated acid and the solid saturated acid fractions by the lead salt method as follows.

The acids were dissolved in a little alcohol, sufficient 2 % aqueous KOH was added to make the mixture neutral to phenolphthalein followed by 15 cc. of a hot 7 % lead acetate solution for every gram of fatty acid present. After cooling for some hours, the aqueous layer was poured off from the precipitated lead salts, which were washed with hot water and drained. These lead salts were then warmed with ether under a reflux until all the particles had been loosened from the sides of the flask, the whole cooled and filtered in an atmosphere of nitrogen, thereby giving ether-soluble and ether-insoluble fractions.

Treatment of ether-soluble lead salt fraction.

The ether-soluble lead salts were decomposed by shaking with dilute HCl and washing the ether solution with water till the washings were free from acid. After drying over sodium sulphate, aliquot portions were taken to determine the weight and iodine value of the acids present and for bromination.

The iodine value determined by Hübl's method was 90, corresponding with that of oleic acid.

The absence of acids of a greater degree of unsaturation, *i.e.* with more than one double bond, was confirmed by the bromination results.

The unsaturated fatty acids freed from moisture and ether were dissolved in sufficient pure carbon tetrachloride to give a 2 % solution and cooled to 0°. To this was added slowly at 0° a 2 % solution of bromine in carbon tetrachloride till the solution was just tinged red. After about 30 minutes an additional slight excess of the bromine solution was run in and the mixture allowed to stand overnight at 0°. The solvent and excess bromine were removed under reduced pressure at laboratory temperature and the residual oil treated with a small amount of dry light petroleum. In no case was there any trace of the precipitation of solid tetrabromide, and a portion of the bromination

product without further treatment was found to contain 35·5 % bromine, corresponding to dibromostearic acid, which contains 35·9 % bromine.

It was found essential to exclude moisture and to allow the bromine to act only in small concentration, otherwise substitution products were formed which caused considerable trouble in the earlier stages of the work. The presence of insoluble hexa- and octo-bromides in the bromination products was never observed although small traces of a white insoluble material containing 14 % Br were found on two occasions; these were not identified.

Treatment of ether-insoluble lead salt fraction.

The insoluble material was treated either with dilute acetic or hydrochloric acid and shaken with ether to extract the liberated fatty acids and the ether solution was washed with water till the washings were neutral. After drying the ether solution over sodium sulphate, aliquot portions were taken for total weight, iodine value, molecular weight and melting-point determination.

It is most important that the I.V. of the "saturated" fatty acids be determined as it is almost impossible to obtain a quantitative separation of saturated fatty acids from unsaturated acids by the lead salt method when dealing with a mixture of a small proportion of saturated with a large proportion of unsaturated acid. A knowledge of the I.V. enables the amount of unsaturated acid still mixed with the saturated acid to be calculated, since oleic acid is the only unsaturated acid found in the lipin.

The molecular weight of the saturated acid was determined after recrystallising the acid from dilute alcohol, dissolving in 96 % alcohol and titrating against $N/10$ sodium hydroxide using phenolphthalein as indicator. Values were found lying between 251 and 260. The recrystallised acid melted at 55–57°.

Palmitic acid melts at 63° and has a molecular weight of 256. Since, however, a specimen of solid acid gave an I.V. of 20, some oleic acid was present and would account for the lowered melting-point and high molecular weight.

Examination of lecithin and kephalin fractions.

Further separation of the lipin into two fractions—the lecithin fraction and the kephalin fraction—was carried out by treating the lipin with excess of absolute alcohol. The residue insoluble in alcohol, which constituted the kephalin fraction, was filtered off and the solution treated with excess of 1 % alcoholic cadmium chloride solution to precipitate the insoluble lecithin-cadmium chloride complex. The compound formed by this addition was completely soluble in ether, yet it still contained one-third of its total nitrogen in the amino-form, indicating that the separation from kephalin was incomplete. The kephalin fraction previously separated by its insolubility in alcohol contained only two-thirds of its nitrogen as amino-nitrogen and was therefore a mixture of kephalin and lecithin in the ratio of 2 : 1. (See Table III.)

The only acids identified from the hydrolytic products of both fractions were palmitic and oleic acids and no evidence of the existence of any other acid was obtained.

The proportion of oleic acid was however greater in the acids derived from kephalin than in those derived from lecithin.

Table IV. *The products of hydrolysis of lipin.*

	Total lipin	Lecithin fraction	Kephalin fraction
Fatty acid %	61·0	64·0	62·0
I.V.	—	64·95	72·45
Fatty acids of ether-soluble lead salts			
(i) Yield %	(a) 53·0	(a) 70·0	(a) 81·0
	(b) 55·0	(b) 70·0	(b) 66·0
(ii) I.V.	(a) 89·97	(a) 75·9	(a) 68·3
	(b) 87·73	(b) 75·9	(b) 83·4
(iii) Br in bromination product %	(a) 39·5	(a) 35·51	(b) 35·42
	(b) 35·5	—	—
Theory for dibromostearic acid ...	35·9		
Fatty acids of ether-insoluble lead salts			
(i) Yield %	(b) 45·0	(a) 30·0	(b) 20
(ii) I.V.	(b) 41·0	54·96	61·9
(iii) Mol. wt.	(a) 251·4	260	259
	(b) 263·0	—	—
(iv) M.P.	(a) 53°	60°	58°
	(b) 54·5°	—	—
Calculated from I.V. since oleic is the only unsaturated acid present			
Unsaturated %	(b) 73·6	72·0	74·5
Saturated %	26·4	28·0	25·5
	100·0	100·0	100·0

As shown in Table IV, the fatty acids of yeast lipins consist of a little palmitic and much oleic acid.

The isolation of the saturated and unsaturated acids in the above experiments was unsatisfactory. The lead salt method of separation is known, however, to work badly when the mixture to be separated contains a large excess of the unsaturated constituent.

The only satisfactory method is by the fractional distillation of the methyl esters but for this a larger quantity of material must be available than we had at our disposal.

Combining the information derived from a knowledge of the I.V. and the amino-N : total N ratio, it is possible to calculate the composition of the yeast lecithin and kephalin, thus:

From nitrogen figures

kephalin fraction contains 62 % kephalin
38 % lecithin

lecithin fraction contains 67·5 % lecithin
32·5 % kephalin

I.V. of mixed fatty acids of

kephalin fraction: 72·45
lecithin fraction: 64·95

Let x be I.V. of mixed fatty acids of kephalin
„ y „ „ lecithin
Then $62x + 38y = 7425$
$32{\cdot}5x + 67{\cdot}5y = 6495$
Hence $x = 82{\cdot}2$
$y = 56{\cdot}55$

As only oleic and palmitic acids appear to be present
I.V. of kephalin acids is due to 91 % oleic acid
9 % palmitic acid
I.V. of lecithin acids is due to 62·6 % oleic acid
37·4 % palmitic acid

These results would suggest that yeast kephalin is a mixture of 82 % dioleyl-kephalin and 18 % oleyl-palmityl-kephalin, and the lecithin a mixture of 75 % oleyl-palmityl-lecithin and 25 % dioleyl-lecithin.

Examination of the "acetone-soluble" fat.

After separation of the lipin, the acetone solution was concentrated, reprecipitated with acetone and the filtered liquid again concentrated under reduced pressure at 40° till free from solvent. The residue consisted of the "acetone-soluble fat." It was a viscid, deep yellow oil which on standing in the cold room deposited white crystals of sterol or sterol ester.

Analysis of this fat showed the presence of only small amounts of phosphorus. The acid value remained fairly constant whereas the saponification value of fat from pressed and incubated yeast showed a distinct difference.

Table V. *Analysis of acetone-soluble fat from*

	(a) Pressed yeast				(b) Incubated yeast		
% P	0·091	0·062	0·048	0·345	0·252	0·075	0·114
A.V.			6·22		7·52	3·26	8·29
S.V.	172	175	176	172	122	128·8	137·2
I.V.			128·1			—	

Hydrolysis of fat.

After saponification of a sample of fat with alcoholic potash in a current of nitrogen, the alcohol was removed by heating on a water-bath and the residue dissolved in water. It was then extracted with ether to remove the unsaponifiable matter, acidified and again extracted to obtain the fatty acids.

(a) *Nature of acids.* As in the case of the lipin examination aliquot portions of the dry ether solution of the fatty acids were taken for iodine value, weight determination and the lead salts separation.

The lead salts soluble and insoluble in ether were separately decomposed as previously described and the liberated acids examined for iodine value, bromination products, molecular weight and melting point.

It was found that *the iodine value of the mixed acids was higher than that of the mixed lipin acids*, and on bromination, di- and tetra-bromo-derivatives were obtained which were separated by taking advantage of the sparing solubility of the tetrabromo-compound in light petroleum. It was found that the proportion of dibromide to tetrabromide was very variable.

Analysis showed that dibromostearic and tetrabromostearic acids were present, hence the existence of oleic and linolic acids in the original fat may be inferred. Palmitic acid was also isolated.

Table VI.

% fatty acid in fat	50·0–60·0 (Mean of 5) 55·1
I.V. of mixed fatty acids	72·75–88·3 (Mean of 6) 77·7
% total fatty acid present as unsaturated acid ...	50·0–56·0 (Mean of 4) 51·5
I.V. of mixed unsaturated acids	97·1–119·6 (Mean of 4) 107·8
% bromine in dibromo-acid	34·95 35·50 36·05
Theory for dibromostearic acid	35·9
% bromine in tetrabromo-acid	53·46
Theory for tetrabromostearic acid	53·33
% total fatty acid present as saturated fatty acid	48·0–50·0 (Mean of 2) 49·0
I.V. of saturated acids	24·0
M.P. of recrystallised acid	55° 57·5° 63°
M.P. of palmitic acid	63°
Molecular weight	249–258 (Mean of 3) 253·7
Theory for palmitic acid	256

(b) *The unsaponifiable fraction.* (See Table VII.) The unsaponifiable material was examined for the presence of ergosterol by warming with light petroleum and recrystallising the soluble material from alcohol. A beautiful white crystalline solid was obtained with an iodine value about 200; M.P. 140–145°. The substance crystallised in characteristic elongated hexagonal plates.

The free sterol in the fat was determined by dissolving 0·4 g. in 50 cc. of 95 % alcohol and adding 50 cc. of a hot 1 % solution of digitonin in 90 % alcohol. After standing a few hours the digitonin-sterol compound was filtered through a Gooch crucible and dried at 110° before weighing. The total sterol was estimated by first hydrolysing 0·4 g. of fat, extracting the unsaponifiable material and precipitating with digitonin as above.

It was found that the fat contained 1 % free ergosterol and some 14 % combined sterol, and that this sterol constitutes 50 % of the total unsaponifiable material.

Since the iodine value of the total unsaponifiable matter is approximately half that of the ergosterol, it follows that the remainder of the unsaponifiable

matter must consist almost entirely of saturated compounds, which we are now further investigating.

Table VII.

	Smedley-MacLean and Thomas [1920]	Smedley-MacLean and Daubney	
% unsaponifiable matter in fat	39·8	24·3–33·35 Mean of 6	29·6
I.V.	—	95·81	
% ergosterol in fat	17·23	15·63	
I.V.	177·1	188·5	200·1
M.P.	—	140–5°	140–5°
% free ergosterol	4·9	1·24	
% combined ergosterol ...	12·3	14·19	
% ergosterol in unsapon. material	43·3	52·7	

Summary and discussion of results.

(1) It has previously been shown that the effect of adding alkali phosphates to a well oxygenated solution of glucose in which yeast is incubated is approximately to double the total amount of ether-soluble (lipoid) substance. At the same time the proportion of lipin is somewhat diminished but the total quantity is much increased. The small amount of nitrogen necessary to form the new lipin must be derived from the nitrogenous material previously existing in the yeast. The phosphorus is presumably derived from the phosphate: the total amount of phosphorus in the yeast is very largely increased and evidence has already been furnished that the phosphate is taken up in association with carbohydrate. No significant variation in the proportion of unsaponifiable matter was observed.

It appears therefore that the addition of phosphate to a sugar solution increases the formation of fat, lipin and sterol and that, as in the animal organism, these lipoid constituents maintain a fairly constant balance.

(2) The phospholipins of yeast consist of lecithin and kephalin and in both of these the same fatty acid radicals, oleyl and palmityl, appear to be present. The proportion of oleyl is apparently greater in the kephalin than in the lecithin molecule. There seems to be some evidence that the proportion of kephalin is higher in lipin from yeast that has undergone partial autolysis than in the freshly produced lipin—a result in accordance with the findings of MacLean for animal tissues. The saponification value (175) of the lipoid matter from the pressed yeast obtained after the yeast had stood for some time in contact with the wort was considerably higher than that (130) of fatty matter from the yeast which had been incubated in the glucose-phosphate medium.

(3) Perhaps the most interesting result established by the experiments now described is that the fatty acids which occur as constituents of the phospholipins of yeast have a lower iodine value than the fatty acids of the acetone-soluble fat. Few reliable data exist for comparing the degree of unsaturation of the acids from the lipins and the acetone-soluble fat respectively of the same organ. The acids of the liver were examined by Kennaway and

Leathes [1909] who found that though the highly unsaturated acids were not confined in the liver to the lipins, yet generally speaking the acids of the liver were more unsaturated than those of the simple glycerides. A similar relation was shown by Bloor [1926] to hold for the fatty acids of the heart. The iodine value of the acids from the heart lipins is very considerably higher than that of the acids from the non-phosphorised fat. Arachidonic acid, a twenty-carbon acid with four double bonds, constitutes 6 % of the lipin acids but only 2 % of the acids from the simple glycerides.

On the other hand, when Bloor [1924] examined the fatty acids which occur in the blood he found that, contrary to expectation, the more highly unsaturated acids for the most part did not occur in the lipins of the blood but were associated with the sterols.

Yeast fat is characterised by the very large proportion of unsaponifiable matter which it contains. This may constitute one-third by weight of the whole fat: approximately half of the unsaponifiable matter consists of the sterol characteristic of the fungi, ergosterol, and nearly the whole of this is in combination with fatty acids as esters. The remainder of the unsaponifiable matter consists of a saturated yellow oil which is being further investigated. In yeast it is the portion of fat containing steryl and glyceryl esters from which the linolic acid is derived. In the yeast lipins oleic acid seems to be the only unsaturated acid present.

(4) Another point of interest about the yeast lipins is that they occur in a less complicated mixture than is usually found in other tissues. In both the kephalin and lecithin fractions from yeast, oleic and palmitic acids appear to be the only two acids present and of these oleic acid is present in very much larger amount. We may conclude therefore that dioleyl- and palmityl-oleyl lecithins and kephalins occur in yeast and that the dioleyl compounds are present in the greater amount. The proportion of the oleic acid is somewhat greater in the kephalin than in the lecithin fraction. In the acetone-soluble fat, on the other hand, saturated and unsaturated acids seem to be present in approximately equal proportions. Very little information is available as to the nature of the acids present in plant lipins. The lipins of the Soya bean were investigated by Levene and Rolf [1925, 1, 2; 1926] who showed that the iodine value of the mixed lipin acids was low compared with that of the mixed lipin acids from animal tissues: the percentage of saturated acid was also exceptionally low. The unsaturated acids isolated from the lipins all contained eighteen carbon atoms and comprised oleic, linolic and linolenic acids. The saturated acids accounted for 15 %, the unsaturated for 43 % and the acids constituting the remaining 42 % were not satisfactorily identified, though some evidence was obtained of the existence of an unsaturated hydroxy-acid. In the work of other observers on various vegetable lipins, oleic acid appears to have been the only acid identified. There are no data available from which a comparison of the acids from the lipins and acetone-soluble fat of the same plant can be made.

On the whole, from such evidence as is available, it seems that the acids of the vegetable lipins have a lower degree of unsaturation than the acids derived from the animal lipins and they are also characterised by a low proportion of saturated acid. In some cases at any rate unidentified acids of unknown structure appear to be present.

Arachidonic acid has not so far been shown to occur in the lipins of the plant kingdom, linolenic acid being the most unsaturated acid yet isolated. Further data as to the nature of the acids present in plant lipins are much needed; a comparison of the acids which occur in the animal and plant lipins may possibly help us to understand something of the functions of the lipins themselves.

Certainly in the yeast lipins, the radicals of highly unsaturated acids seem to be absent and the doubly unsaturated acid of yeast fat occurs only in combination with sterol or glycerol.

We desire to express our indebtedness to the Food Investigation Board of the Department of Scientific and Industrial Research for grants which have enabled us to carry out this work: our thanks are also due to Miss E. Clenshaw for her assistance in carrying out part of this work and to the Directors of Messrs Watney, Coombe, Reid and Co. for supplying the yeast for this investigation.

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LV. NOTE ON SOME STEROL COLOUR REACTIONS IN THEIR RELATION TO VITAMIN A.

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The discovery of the brilliant blue colour reaction of cod-liver oil with $AsCl_3$ and its suggested association with both vitamin A and cholesterol [Rosenheim and Drummond, 1925] led to a search for a similar reaction, obtainable from sterols or their derivatives under conditions which would throw light on the nature of the chromogen concerned in the cod-liver oil reaction. Starting from the assumption that oxidative processes are connected with the formation of the blue pigment in sterol reactions, the action of mild oxidising agents on sterols was investigated. It was found that treatment of cholesterol with benzoyl peroxide in chloroform solution produced a chromogenic substance which gave with $AsCl_3$ a blue colour indistinguishable in appearance from that given by cod-liver oil with $AsCl_3$. The possibility of the formation of an aldehydic sterol derivative on mild oxidation suggested further a study of the influence of formaldehyde on the colour reactions of sterols. Again a change from the usual red colour to a clear blue was found to be produced when $AsCl_3$ reacted with cholesterol in the presence of nascent formaldehyde. This reaction is conveniently carried out by adding $AsCl_3$ to a solution of cholesterol in methylal. In this case, however, the chromogen cannot be isolated, as it is slowly converted into the pigment in the course of the reaction[1].

Although the blue pigment formed from cholesterol resembles on superficial inspection that obtained in the cod-liver oil reaction, a more detailed investigation raised doubts as to their identity. This doubt was increased when it was found that cholic acid also gives rise to a similar blue colour reaction after heating with benzoyl peroxide in chloroform solution. The latter reaction again demonstrates the near relationship of the bile acids to cholesterol, the nature of which has been cleared up by the work of Windaus and Neukirchen [1919]. The similarity of these colour reactions to that of the chromogen of cod-liver oil suggests that there exists a similarity between the carbon ring

[1] Antimony trichloride, trichloroacetic acid, dimethylsulphate, etc., may be used as condensing agents in place of $AsCl_3$ in all these reactions under slightly modified conditions. The various phytosterols and ergosterol react in a similar way. In view of the multiplicity of sterol reactions already available, a detailed description of the new reactions may be dispensed with.

system of the sterol molecule and that of the unknown chromogen and lends support to the view that oxidative changes of the sterol molecule may be concerned in the vitamin A formation from sterols.

Evidence is accumulating indicating that the blue colour reaction of cod-liver oil is specific for vitamin A [Rosenheim and Webster, 1926, 1927, 1]. As the new colour reactions of sterols are of interest only in their relation to this vitamin, a short description of one of them is sufficient in this respect. The chromogen is rapidly formed on evaporating to dryness a chloroform solution of cholesterol and benzoyl peroxide in equimolecular proportions. A quantitative colorimetric test in Lovibond's colorimeter showed that the maximum colour production is reached after boiling the solution for about two hours (1 mg. of the reaction product + 2 cc. $SbCl_3$ solution equals 12 blue units). All attempts to isolate the chromogen in a pure condition from the complex mixture of amorphous oxidation products have so far failed.

The properties of the resinous product obtained resemble those described for a similar mixture called "oxycholesterol" by Lifschütz, and like the latter material it can also conveniently be prepared from cholesterol dibromide by debromination with sodium acetate in alcoholic solution [Lifschütz, 1919]. The substance to which Lifschütz ascribes, without justification, the formula $C_{27}H_{46}O_2$, is undoubtedly a complex mixture [see Windaus, 1908; Windaus and Lüders, 1921; Gardner, 1921], one constituent of which gives the green colour reaction, considered as characteristic for "oxycholesterol" by Lifschütz. The material also gives a blue reaction with $AsCl_3$[1], but it appears doubtful whether the constituent giving rise to the green Lifschütz reaction is identical with the chromogen of the blue pigment. In any case there can be no doubt that Lifschütz's "oxycholesterol" has no bearing on the blue cod-liver oil reaction with $AsCl_3$, since, according to Lifschütz's and my own observations, the typical green colour reaction is not given by any liver fat (nor by cod-liver oil).

On the other hand, the fact that one constituent of the oxidation products of cholesterol gives a blue colour with $AsCl_3$ suggested an investigation of its behaviour when added to such vegetable or animal oils as do not react with $AsCl_3$ by themselves. It was found that in every case examined the production of the blue colour was completely inhibited when a purified preparation (from cholesterol dibromide) was dissolved in any natural oil or fat. This instability of the artificial chromogen in solution in a natural oil excludes its identity with the chromogen of cod-liver oil. The rapid destruction of the artificial chromogen appears to be connected with the presence of unsaturated linkages in the natural oils, for a freshly prepared solution in a neutral solvent, such as liquid paraffin, gives an intense positive reaction with $AsCl_3$. Even in this case, however, the chromogen gradually disappears on keeping under laboratory conditions.

[1] In the course of an examination of the sterols of echinoderms [Page, 1923], and of *Boletus granulatus* [Marston, 1924], the same observation is recorded incidentally.

Although in its relative instability the artificial chromogen resembles the chromogen of cod-liver oil, it may be pointed out that the blue pigment produced by $AsCl_3$ from the cod-liver oil chromogen is typically unstable, the colour fading within a few minutes, whilst the blue colour given by the artificial product, when once formed, remains unchanged for many days. A characteristic difference in the absorption spectrum of the two pigments may be adduced as a further reason against their identity.

A few feeding experiments with the mixture of substances obtained on oxidation of cholesterol were kindly carried out by my colleague, Mr Webster. The results so far obtained, although not yet completely conclusive on account of the instability and impurity of the material, are against the view of its having the properties of vitamin A. In consideration of the relationship which has recently been shown to exist between ergosterol and the antirachitic vitamin D [Rosenheim and Webster, 1927, 2; Windaus and Hess, 1927], we are at present investigating the possible relation of vitamin A to the oxidation products of ergosterol.

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LVI. THE PARENT SUBSTANCE OF VITAMIN D.

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Two years ago we found that cholesterol, having the characteristics then known of the pure substance, was rendered antirachitic by exposure to ultra-violet light [Rosenheim and Webster, 1925]. This fact was also discovered independently, and at about the same time, by the American observers Steenbock and Black [1925] and Hess, Weinstock and Helman [1925].

Although this discovery has since been confirmed and accepted by numerous investigators in many countries, and has found practical application for the cure of rickets in children, we were able to show last year [Rosenheim and Webster, 1926, 1] that cholesterol when purified still further by a chemical method had lost the property of becoming antirachitic by irradiation. In agreement with this observation, it was found that chemically purified cholesterol no longer possesses the absorption spectrum in the ultra-violet region which is characteristic for cholesterol purified by physical means only.

These facts threw new light on the photo-chemical formation of vitamin D. It became evident that the precursor of vitamin D (= provitamin) is not cholesterol itself, but an unknown substance associated with cholesterol as obtained from all natural sources (gallstones, brain, eggs, etc.)[1].

The fact that the presence of an impurity in such a well crystallised substance as cholesterol has for more than a hundred years escaped recognition by chemists is explained by the minute amount (1:2000) concerned, and emphasises the delicacy of the biological test. It may be pointed out that the same method which enabled Reinitzer [1888] to arrive at the correct empirical formula of cholesterol, *i.e.* bromination, has now led also to the discovery of the biologically pure cholesterol.

The newly established facts proved valuable for the rapid progress of the work on the nature of the provitamin, in so far as they enabled us to rely, to a large extent, on the spectroscopic test in place of the tedious animal experiment. Although such a test had already been tentatively suggested by Schlutz and Morse [1925], a firm basis for its application was only established when we showed by animal experiments that the absence of absorption bands

[1] From the historical point of view, it is interesting to note that the suggestion of an impurity in cholesterol as the causative factor concerned in its activation by ultra-violet light had already been considered by Steenbock and Black [1925] and by Schlutz and Morse [1925], the latter observers basing themselves on spectrographic observations only.

in the ultra-violet coincided with the loss by cholesterol of its property of becoming antirachitic on irradiation. It is evident that the destruction or absence of provitamin can be rapidly proved by a negative spectroscopic test, whilst the animal experiment remains indispensable in confirmation of positive spectroscopic evidence.

In the search for the nature of the provitamin we had the privilege of collaborating with Prof. Windaus (Göttingen). Separate preliminary communications on this part of the work, the outcome of mutual suggestions, were made simultaneously by Prof. Windaus [Windaus and Hess, 1927], and by ourselves [Rosenheim and Webster, 1927, 1], according to a friendly arrangement.

In the first instance we established the fact that not only cholesterol but the vegetable sterols also could be freed from the provitamin by bromination. It was found that stigmasterol ($C_{30}H_{48}O$), containing two double bonds, and sitosterol ($C_{27}H_{46}O$), with one double linkage, when purified by way of the bromides, also yield no vitamin on irradiation; whereas all vegetable oils, and the sterols obtained therefrom and not so purified, readily acquire antirachitic properties under the action of ultra-violet rays.

Ordinary cholesterol may be freed from provitamin, as we have since discovered, not only by bromine, but also by various other chemical as well as physical methods. Thus pure cholesterol may be prepared from cholesteryl chloride, which cannot be activated itself, by conversion into cholesterol acetate [Mauthner and Suida, 1894] and subsequent saponification. Treatment of cholesterol in acetone solution by potassium permanganate, whilst leaving cholesterol unattacked, removes the provitamin by oxidation. Of the physical methods the simplest consists in over-irradiation and subsequent recrystallisation. Further, repeated treatment with an efficient charcoal frees ordinary cholesterol from provitamin, but whether this is due to adsorption or oxidation has not yet been investigated.

Whilst these results afforded no means for the isolation or even concentration of provitamin, more direct evidence as to its nature was furnished by the observation that, unlike the vitamin obtained from it, provitamin forms an addition compound with digitonin. Although the product recovered from the digitonide of ordinary cholesterol by fractional extraction with xylene contains provitamin in increased amount, the complete separation of the latter from cholesterol or other sterols by this method does not appear to be practicable.

These observations afford significant information with regard to the chemical nature of provitamin. We may conclude that it is destroyed by oxidation and by bromine, and that it possesses the properties of a sterol, in so far as it forms an addition product with digitonin. The sensitiveness of provitamin to oxidative processes is a characteristic of only one of the known sterols, *i.e.* the highly unsaturated ergosterol ($C_{27}H_{42}O$). Its discoverer, Tanret [1890, 1908] had already studied the oxidation of ergosterol under the influence

of light and air, as evidenced by its change in colour, fall of melting point and optical rotation [see also Schulze and Winterstein, 1906]. Two other properties of provitamin are characteristic also for ergosterol, namely its destruction by bromine[1] and the formation of an insoluble digitonide [Smedley-MacLean and Thomas, 1920].

This similarity in their known properties suggested to Prof. Windaus that provitamin may be nearly related to, if not identical with, ergosterol and we therefore took up again, at his suggestion, the study of ergosterol which we had previously found [Rosenheim and Webster, 1926, 2], after irradiation "to be highly protective even in doses of one milligram" when comparing it under identical conditions with 5 mg. doses of irradiated cholesterol on the same batch of rats.

In view of the more recent work on the absorption spectrum of cholesterol by Heilbron, Kamm and Morton [1926] and by Pohl [1926], we compared the absorption spectrum of ergosterol with that of a specimen of ordinary cholesterol. One would expect ergosterol, with its three double bonds, to show a greater absorption in the ultra-violet region than cholesterol, which has only one double linkage. It was, indeed, found that ergosterol exhibited a very pronounced absorption in the ultra-violet region, the characteristic bands of absorption being identical with those of cholesterol but of enormously greater intensity. The intensity of the absorption was estimated to be 1500–2000 times as strong as that of brain cholesterol[2]. As the result of the irradiation the absorption bands disappear just as in the case of cholesterol; at the same time the product loses its property of being precipitated by digitonin. If provitamin were ergosterol, or an unsaturated sterol of similar constitution, the amount present in ordinary cholesterol would be of the order of 0·05 %, or one part in 2000, and irradiated ergosterol should in this case possess antirachitic activity in correspondingly small doses.

These assumptions have been fully confirmed by biological tests on rats. A daily dose of 1/10000 mg. of irradiated ergosterol cured and prevented rickets in rats kept on a rachitogenic diet. Tests with still smaller quantities, not yet completed, indicate that the limit of activity will prove to be less than 1/50000 mg. Irradiated ergosterol is therefore the most potent antirachitic substance known, 5 mg. being equivalent to approximately 1 litre of a good cod-liver oil.

It is of interest to note that a dose 10000 times greater than what is at present regarded as an effective dose, produced no obvious ill effects on rats under the experimental conditions used.

These results seem to justify the conclusion that the naturally occurring

[1] See also Rosenthal [1922]. According to previous unpublished observations of Windaus, ergosterol cannot be recovered unchanged from its amorphous bromination products.

[2] Since the provitamin content of cholesterol, purified by physical methods, is variable (see later) this figure necessarily varies with different specimens and may be as high as 1:10,000 or more.

parent substance of vitamin D is ergosterol or a sterol possessing the same absorption spectrum and physiological activity. The admixture in cholesterol from all animal sources (brain, eggs, gallstones, etc.) of a sterol with more than one double bond seems likely, in view of the well-known occurrence in phytosterols from vegetable oils, seeds, etc., of other representatives of this class, containing two double bonds. In this connection the recent work of Anderson [1927] is of interest; he drew attention to the fact that cholesterol preparations obtained from different sources show differences in physical properties, which are ascribed to the probable admixture of isomeric or different sterols. Their partial separation from cholesterol should be possible not only by fractional distillation in a high vacuum, as found by Windaus, but also by fractionation in superheated steam [see the suggestive results of Gardner, 1921].

It should be added that although ergosterol was originally isolated by Tanret from ergot, and named accordingly, similar or identical sterols ("mycosterols") have been obtained from a wide range of the lower plants, occurring, for example, abundantly in the fat extracted from certain yeasts [Smedley-MacLean and Thomas, 1920; Windaus and Grosskopf, 1922]. The ergosterol employed in our own experiments above quoted was actually prepared from ergot, whilst that used by Prof. Windaus was obtained from yeast.

The action of ultra-violet light on ergosterol leads to an obvious physical change and the production of a yellowish resin. The nature of the intramolecular change which gives rise to the vitamin formation is at present unknown.

Experimental.

Methods. The irradiation of the substances to be tested, and the feeding experiments, were carried out by the technique described in our former communications. Negative controls with the basal diet only, as well as positive controls (basal diet + 0·1 cc. cod-liver oil), were made in every set of experiments, although not specifically reported in the tables. For the diagnosis of rickets we relied on the evidence of the radiograph and the estimation of the content of inorganic phosphates in the blood. The large number of radiographs necessary for this work were again kindly taken for us in the department of our colleague, Mr J. E. Barnard, F.R.S., to whom we wish to express our best thanks. It might be mentioned that the results were in all cases decisive, the rats showing either severe rickets or complete absence of rickets.

I. *Removal of the provitamin from various sterols.*

(1) *Phytosterols.* The method originally worked out for the separation of stigmasterol from other phytosterols by Windaus and Hauth [1906] depends on the insolubility of the tetrabromide of stigmasterol acetate. The necessity of using bromine in its preparation explains, in the light of our present knowledge, the previously surprising result that this naturally occurring sterol could not be activated. It may be pointed out that even the presence of two unsaturated linkages in a sterol is apparently not sufficient to enable it to

act as a provitamin. Stigmasterol (M.P. 170°) irradiated in air for one hour and administered in daily doses of 2 mg. and 4 mg. was unable to protect rats against rickets (see Table I).

Table I.

Substance	Stigmasterol	Sitosterol	Cholesterol recovered from over-irradiated ordinary cholesterol	Olive oil; fraction of "unsaponifiable" not precipitated by digitonin	Cholesterol from ordinary cholesterol-digitonide	
					Fraction (1)	Fraction (2)
Time of irradiation	1 hr.	1 hr.	30 min.	30 min.	30 min.	30 min.
Dose	2 mg.	2 mg.	2 mg.	40 mm.³	2 mg.	2 mg.
	4 mg.	4 mg.	4 mg.	40 mm.³ 160 mm.³	4 mg.	4 mg.
Radiographic result	Rickets	Rickets	Rickets	Rickets	Normal	Normal

The dibromide of sitosterol acetate is soluble and remains in the filtrate from the stigmasterol derivative. Sitosterol itself may be prepared, without previous isolation of its bromide, from the above filtrate by the method described by Windaus and Hauth [1907].

The specimen of sitosterol (M.P. 136–137°) used in the feeding test was prepared from the phytosterols of Soja beans by the above method and when tested biologically in the same way as stigmasterol was found to be inactive (see Table I). Both sterols showed absence of absorption bands in the spectroscopic test.

(2) *Cholesterol.* The bromination method has already been described in our former communication [1927, 2].

(*a*) *Thionyl chloride* (or phosphorus pentachloride) may be used instead of bromine for the removal of provitamin. Cholesteryl chloride is obtained in the first instance and is subsequently converted into the acetate by boiling with zinc dust in acetic acid solution [Mauthner and Suida, 1894]. Cholesterol was recovered by saponification of the acetate, and gave a negative spectroscopic test.

It may be mentioned in explanation of this result that ergosterol is apparently destroyed by PCl_5 [Reindel, Walter and Rauch, 1927].

(*b*) *Over-irradiation* destroys vitamin D, as has been previously shown. By converting all the provitamin present in cholesterol into vitamin D by irradiation, and subsequently destroying it by over-irradiation, it is possible to recover pure cholesterol after removal of the reaction products by recrystallisation. From an experiment devised to ascertain the possibility of reactivating cholesterol which had been deactivated by over-irradiation in the dry state, Hess, Weinstock and Sherman [1925] drew the conclusion that a substance "quite different from the original cholesterol had been formed." We found it necessary to irradiate a solution, and not the crystals, of ordinary cholesterol in order to induce the complete destruction of both the provitamin and the vitamin.

The substance was dissolved in ether and irradiated in a quartz flask for 10 hours. After evaporation of the ether, the cholesterol was recrystallised from alcohol and the whole process repeated. The final product was again recrystallised, irradiated for 30 minutes, and administered to rats in daily doses of 2 mg. and 4 mg. (see Table I). The spectroscopic, as well as the biological test, gave negative results.

(*c*) *Charcoal* easily removes the provitamin from ordinary cholesterol. Five g. cholesterol were dissolved in 50 cc. 96 % alcohol and boiled with 1 g. norit for half an hour, under reflux. The filtrate was treated again with 1 g. norit. The recovered cholesterol was found to be free from provitamin by the spectroscopic test (see Fig. 1, Plate I). The same result was obtained by using Merck's blood charcoal.

In view of the variable content in provitamin of commercial samples of cholesterol, possibly purified by means of charcoal, caution is indicated against the indiscriminate use of irradiated cholesterol as a source of vitamin D, when testing for the presence of vitamin A. We experienced a failure, unexplained at the time, of a series of feeding experiments dealing with vitamin A estimations owing to the employment of a cholesterol prepared from cod-liver oil by ourselves. The specimen in question had been carefully purified by charcoal in order to adsorb the persistent yellow pigments, which could not be removed by recrystallisation alone. We have since come across two commercial samples of cholesterol equally deficient in provitamin. We propose to use a solution of irradiated ergosterol in future work on vitamin A.

(*d*) *Potassium permanganate* in acetone solution may also be used. The procedure need not be described in detail, and it is sufficient to state that cholesterol obtained on concentration of the solution (after the filtration from MnO_2) is spectroscopically inert.

II. *Precipitation of provitamin by digitonin.*

We obtained decisive evidence for the formation of a digitonide of provitamin in the first instance by an indirect route. Olive oil (200 cc.) was saponified in the usual way. The unsaponifiable fraction could easily be activated by means of irradiation, as shown by a separate experiment. The alcoholic solution of the unsaponifiable fraction (0·64 g.) was precipitated with excess of digitonin. The filtrate from the digitonide, after evaporation *in vacuo*, was taken up in water and extracted with ether. The ether-residue was irradiated for 30 minutes, dissolved in 3 cc. liquid paraffin and administered in daily doses of 40 $mm.^3$ and 160 $mm.^3$ These amounts, corresponding to 2·5 cc. and 10 cc. of irradiated olive oil, were unable to prevent severe rickets, thus proving that provitamin had been completely removed by digitonin (see Table I).

Direct evidence of the presence of provitamin digitonide in ordinary cholesterol digitonide was furnished by precipitating ordinary cholesterol (from gallstones) with digitonin. The digitonide was recrystallised from

pyridine-alcohol, washed with ether and dried. The product was fractionally extracted with boiling xylene, fraction (1) representing cholesterol obtained after the first eight hours' extraction. The extraction was continued for another eight hours and the product labelled fraction (2). Spectroscopic examination of fraction (1) showed a considerable increase of absorption in the ultra-violet, indicating about double that of the original cholesterol. The animal experiment proved that both fractions prevented rickets after irradiation, when administered in daily doses of 2 mg. and 4 mg. (see Table I). No attempt was made to assess the difference in antirachitic activity of the two fractions by the animal experiment.

III. *Ergosterol as provitamin D.*

The specimen of ergosterol used in this work was part of the same preparation, kindly put at our disposal by Dr H. A. D. Jowett, which we had previously found to be easily activated [1926, 2]. It had been prepared from ergot and since it possessed the constants given by Tanret [1908] for pure ergosterol, M.P. 162°, $[\alpha]_{5461}^{19°} = -127°$, must be considered as free from fungisterol.

(1) *Spectroscopic evidence.* The absorption spectra were photographically recorded by means of a Hilger's quartz spectrograph, using as a source of light a quartz mercury vapour lamp. An all-quartz cell of 10 mm. width was used, pure ether (free from peroxides) serving as the solvent.

The absorption of ultra-violet light by ergosterol was found to be enormous, even a solution of 1 : 2000 absorbing the whole of the region from 310$\mu\mu$ onwards (see 5, Fig. 1, Plate I). Many photographs were taken in which the absorption spectra of a series of dilutions were recorded, the exposure being kept constant. In Fig. 2 (Plate I) the strength of the solutions varied from 1 : 5000 (1) to 1 : 50000 (10), even this extreme dilution still showing distinct absorption between 280–310$\mu\mu$. It is interesting to note that the absorption extends well into the ultra-violet region of sunlight, which is able to activate ordinary cholesterol, as we have previously shown.

In Fig. 1 (2) is shown for comparison the absorption spectrum of a solution 1 : 10 of ordinary cholesterol. The removal of the provitamin by charcoal treatment is illustrated by Fig. 1 (3), whilst (4) brings out the striking influence of the addition of ergosterol 1 : 20000, to a solution of pure cholesterol 1 : 10. By comparing (2) and (4) it will be seen that the absorption in the critical region of from 280–310$\mu\mu$ is almost identical, and the conclusion may be drawn that the absorption in the ultra-violet of ergosterol is about 2000 times that of ordinary cholesterol. This merely qualitative judgment was confirmed by quantitative measurements made in Göttingen (see footnote, p. 391).

Although the absorption of pure cholesterol in the ultra-violet is powerfully affected by the addition of this minute amount of ergosterol, it might be mentioned that no change in physical properties, such as melting point and

specific rotatory power which usually serve to the chemist as a criterion of purity, can be demonstrated in an artificial mixture of pure cholesterol containing 0·05 % ergosterol. The admixture of ergosterol cannot be detected by the usual colour reactions, since no specific colour reaction for it in the presence of other sterols is so far known.

The gradual disappearance of the characteristic absorption bands, which takes place on irradiation, may be taken to coincide with the formation of vitamin D, which is also brought into evidence by the coincident decrease of digitonin-precipitability. By taking a series of absorption spectra after exposing a 1 : 1000 ergosterol solution to the prolonged action of ultra-violet light, it was found that an exposure of approximately five hours was necessary to cause the complete "bleaching out" of the absorption. After the same period the product did not possess the property of forming an insoluble digitonide.

(2) *Biological evidence.* Two main series of experiments were carried out. In the first, ergosterol was irradiated as a solid in air for 30 minutes, and in the second series a solution in pure ether was irradiated. A quartz cell ($10 \times 25 \times 25$ mm.) with a narrow tubular neck was employed in order to restrict the exposure of the solution to air as much as possible. The ether solution was irradiated in the closed cell for 30 minutes and 5 hours respectively. After evaporation of the solvent, the irradiated material was dissolved in olive oil, which had previously been tested for the absence of vitamin D by the animal experiment. The daily doses varied from 0·025 mg. to 0·0001 mg. (= 1/40 to 1/10000 mg.). The results were identical in all cases, *i.e.* complete protection and cure were effected even by the smallest daily dose of 1/10000 mg. (see Table II). Experiments are in progress with still smaller doses. We have reason to believe that the limiting dose will be considerably less than 1/50000 mg. when the irradiation is carried out under the most favourable conditions in the absence of oxygen.

Table II.

Substance	Ergosterol (in ethereal solution)		Ergosterol (as a solid)
Time of irradiation	30 minutes	5 hours	30 minutes
Dose in mg.	1/40, 1/80, 1/500, 1/1000 1/10000	1/40, 1/80, 1/500, 1/1000 1/10000	1/40, 1/80, 1/125, 1/250, 1/500, 1/1000 1/10000
Radiographic result	Normal	Normal	Normal

An interesting proof of the stability of irradiated ergosterol is afforded by the fact that a solution in an inactive oil kept for $1\frac{1}{2}$ years was found to be still active when tested in daily doses of 1/1000 mg. This solution had served for our original work on the activation of ergosterol and had been kept in a closed flask in a cupboard.

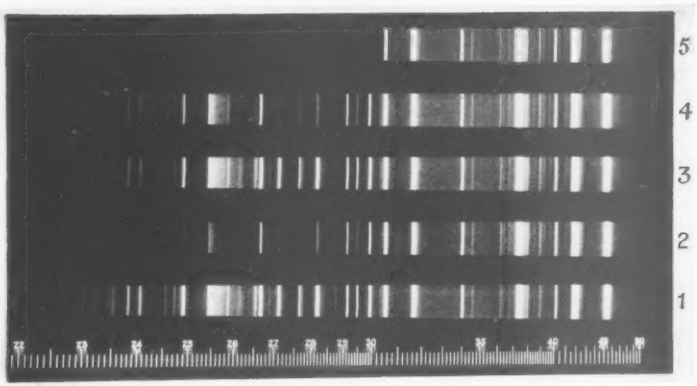

Fig. 1.

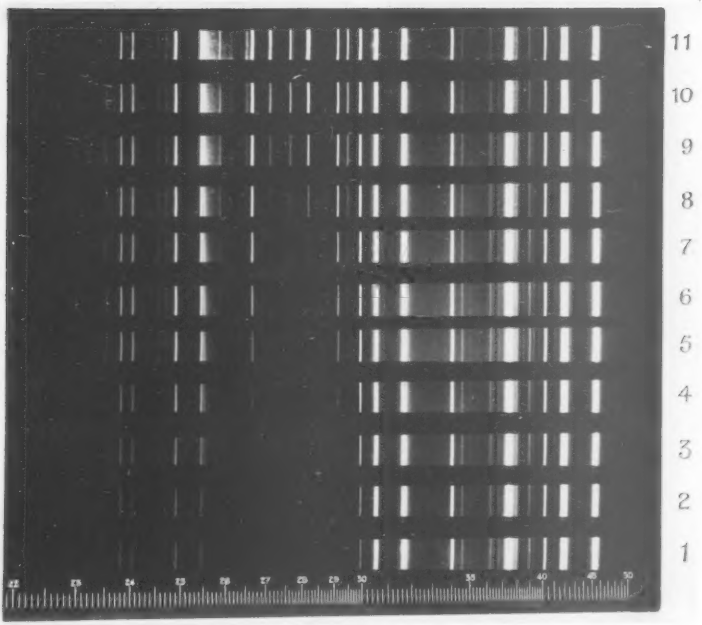

Fig 2.

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SUMMARY.

(1) Cholesterol and phytosterols, purified by the methods described, can no longer be rendered antirachitic by ultra-violet light.

(2) The nature of the impurity present in ordinary sterols which is responsible for their activation by ultra-violet light has been investigated. The evidence obtained leads to the view that the impurity is a sterol of an unsaturated and labile type, of which ergosterol is the only known representative.

(3) Ergosterol was found to possess the same characteristic absorption spectrum in the ultra-violet as non-purified cholesterol, the intensity of the absorption, however, being enormously increased. Assuming it to be ergosterol, the amount of impurity present in ordinary cholesterol was judged by comparative spectroscopic examination to be of the order of 1 : 2000.

(4) Irradiated ergosterol in daily doses of 1/10000 mg. cures and prevents rickets in rats on a rachitogenic diet.

(5) It is concluded that the natural parent substance of vitamin D is ergosterol, or a highly unsaturated sterol of similar constitution, which is converted into vitamin D by irradiation.

EXPLANATION OF PLATE I

Fig. 1. Comparative absorption spectra of cholesterol and ergosterol in solution in ether.
(1) Ether. (2) Ord. cholesterol in ether, 1 : 10. (3) Same after charcoal treatment.
(4) Pure cholesterol, 1 : 10, plus ergosterol 1 : 20000. (5) Ergosterol, 1 : 2000.

Fig. 2. Absorption spectra of ergosterol.
(1–10) Ergosterol in ether, 1 : 5000 to 1 : 50000. (11) Ether.
Source of light: quartz mercury lamp.
Width of cell: 10 mm.

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LVII. THE INFLUENCE OF AMINO-ACIDS ON HYDROLYSIS BY PANCREATIC LIPASE.

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The effect of amino-acids on pancreatic lipase has been studied as a preliminary to a general investigation of the influence of proteins on hydrolysis by this enzyme, following results previously reported that certain proteins may influence the mode of action of lipase. It was found [Platt and Dawson, 1925] that the enzyme exerts preferentially an "esterase" or "lipase" action when associated with albumin and globulin respectively.

Amino-acids have been shown to increase the action of certain enzymes; Jacoby and Umeda [1914] found that glycine, alanine, leucine, tyrosine and glutamic acid accelerate hydrolysis by soya-bean urease. This promoter effect is apparently specific for α-amino-acids. Kato [1923], using glycine as a typical amino-acid, investigated the phenomenon in more detail and eliminated any possible effect due to the buffer value of the added amino-acid by conducting the hydrolysis in the presence of a phosphate buffer mixture. He suggests that a definite chemical combination between glycine and urease is indicated.

Sherman and his co-workers have made exhaustive investigations on the influence of amino-acids on various amylase preparations. All α-amino-acids tested were found to accelerate the hydrolysis of starch by amylase, while other compounds containing an amino-group, carboxyl group or both had no influence or even an inhibitory effect on the enzyme. Sherman and Walker [1923] advance the hypothesis that the apparent accelerating power of amino-acids is due to their retarding the destruction of the enzyme, to which they ascribe a protein structure. In support of this they have shown that the amino-acids have a relatively greater action at higher temperatures, when the destruction of the enzyme by hydrolysis will be greater.

Falk and Nelson [1912] have stated that amino-acids and products of protein hydrolysis are capable of hydrolysing esters. An analysis of their results shows that the actual amounts of hydrolysis observed are small, while sufficient attention does not appear to have been given to the control of the hydrogen ion concentration. In preliminary experiments, I carried out control estimations in which ester and amino-acid were incubated without the addition of enzyme. When the change in hydrogen ion concentration, due to the addition of amino-acid to ester and buffer or ester and water mixtures, was carefully controlled, no hydrolysis of ester due to amino-acid alone could be

demonstrated. This result has been confirmed by Bosman [1926], who repeated some of Falk's experiments and proved conclusively that any hydrolysis of ester which does occur is due, not to any specific action of the amino-acids, but to the acidity developed in the medium on the addition of these compounds.

With regard to previous work on the influence of amino-acids and related substances on lipase, Magnus [1904] was unable to activate an inactive preparation of liver lipase by the addition of glycine, alanine and tyrosine, while Terroine [1920] stated that glycine and Witte's peptone have no influence on the lipolytic action of pancreatic juice. Willstätter and Memmen [1923], however, observed a small increase in the rate of hydrolysis of tributyrin by pancreatic lipase on the addition of alanine, and a large increase on the addition of the tripeptide leucylglycylglycine. The hydrogen ion concentration was controlled by adding an ammonia-ammonium chloride buffer solution to the digestion mixtures.

Experimental.

The enzyme was used either in the form of a powder prepared by treatment of pig's pancreas with acetone and ether, or as a clear glycerol extract obtained by digesting the powder with 87 % glycerol and filtering through a hardened filter-paper. Ethyl butyrate and olive oil were chosen as substrates. When using the former the activating power on an amino-acid was obtained by determining the number of cc. of $N/10$ NaOH required to neutralise the acid formed from 0·5 cc. of ester in the presence and absence of amino-acid. The amino-acids were added from freshly prepared solutions adjusted to the correct p_H, the final concentration of amino-acid in the digestion mixture being 0·01 M.

The amount of hydrolysis of olive oil was estimated by the technique of Willstätter and Waldschmidt-Leitz [1923]. The titration with alcoholic potash was carried out in an alcohol-ether mixture using thymolphthalein as indicator. Amino-acids were added to the control estimations immediately before titration, in order to correct for the titration values of these substances in alcoholic solution.

Hydrolysis of ethyl butyrate.

The first experiments with the ethyl butyrate were carried out in the presence of a phosphate buffer mixture p_H 7·0. It was found that the increase in hydrolysis due to amino-acids under these conditions was small, owing to the very marked activating power of phosphates on lipase. The estimations on ethyl butyrate were, therefore, made without the addition of buffer solutions, the reaction mixtures being adjusted to p_H 7·6 initially.

Exp. 1. Mixtures were made up containing

10 cc. water
2 cc. amino-acid solution
2 cc. suspension containing 10 mg. of enzyme
0·5 cc. ethyl butyrate.

These were incubated with continuous shaking for three hours at 37°. The results are the mean of duplicate experiments corrected for the suitable controls.

Amino-acid added (0·01 *M*)	Mean titration value cc.
—	2·25
Glycine	2·60
Alanine	2·60
Leucine	2·65
Aspartic acid	3·25
Glutamic acid	3·90
Histidine	5·35

Exp. 2. Conditions as in Exp. 1 except that 0·5 cc. of glycerol extract 1 was used as source of enzyme.

Amino-acid added	Mean titration value cc.
—	0·88
Glycine	1·37
Alanine	1·33
Leucine	1·35
Aspartic acid	1·90
Glutamic acid	2·35
Histidine	4·10
Lysine	1·88
Ornithine	2·00

The effect of the amino-acids is more marked when the glycerol extract is used. This is probably because protein activating substances have been removed by this further purification.

Hydrolysis of olive oil.

Exp. 3. Mixtures were made up consisting of

2 cc. ammonia-ammonium chloride buffer p_H 9·2
2 cc. solution containing 10 mg. calcium chloride
2 cc. amino-acid solution
2 cc. suspension containing 10 mg. enzyme
4 cc. water
2·5 g. olive oil.

These were shaken for three minutes and incubated at 37° for a further 57 minutes. The results are expressed as the percentage of the oil hydrolysed and are the mean of duplicates corrected for controls.

Amino-acid added	% hydrolysis
—	12·7
Glycine	13·8
Aspartic acid	18·7
Glutamic acid	19·3
Arginine	12·4
Lysine	19·6
Tryptophan	13·3
Phenylalanine	14·5

Exp. 4. Conditions as in Exp. 3 except that 1 cc. of glycerol extract 2 was used.

Amino-acid added	% hydrolysis
—	19·8
Glycine	22·45
Alanine	22·55
Leucine	22·55
Aspartic acid	26·7
Glutamic acid	27·9
Histidine	32·5

Exp. 5. Conditions as above using 0·5 cc. glycerol extract 3.

Amino-acid added	% hydrolysis
—	13·7
Aspartic acid	21·0
Proline	15·9
Tyrosine	16·1

A consideration of the above data shows that all the amino-acids tested, with the exception of arginine, accelerate the hydrolysis of ethyl butyrate and of olive oil by pancreatic lipase. The simple monoamino-monocarboxylic acids have small effects which are equal in magnitude, while histidine has the greatest promoter action of any of the substances tested. The acceleration of hydrolysis is not due to a change in the hydrogen ion concentration on the addition of amino-acid, since the p_H of the solutions was carefully controlled by colorimetric and electrometric observations.

It was found, however, that amino-acids do not influence hydrolysis by lipase in acid solution. This was shown by a similar series of experiments to those reported above, in which acetate buffer mixtures at p_H 5·4 for ethyl butyrate and p_H 4·7 for olive oil replaced the alkaline buffer solutions. This result suggests that amino-acids accelerate hydrolysis by stabilising the enzyme in neutral or alkaline but not in acid solutions, and a certain amount of experimental evidence has been obtained which confirms this view.

In the first place it was observed that the promoter effect of the added amino-acid is greater at higher temperatures. Thus the addition of 0·01 *M* aspartic acid raises the optimum temperature for the hydrolysis of olive oil by lipase from 37° to 44°, when the estimations are carried out in the usual manner in the presence of alkaline buffer mixtures and calcium chloride.

This indicates that amino-acids retard the destruction of the enzyme by hydrolysis, since such a decomposition, which involves the splitting up of either the lipase or of associated substances upon which the stability of the enzyme depends, will normally be greater at higher temperatures. If this explanation is correct, the enzyme when heated in the absence of substrate should retain its lipolytic activity longer when the incubation is carried out in the presence of added amino-acid than when heated in aqueous or buffer suspensions alone. To test this point mixtures of enzyme and buffer solution were made up as for the estimation of hydrolysis of olive oil; aspartic acid or histidine was added to one series, and all were incubated at 37° before adding the substrate. Aspartic acid or histidine was then added to those mixtures which did not already contain amino-acid, and the amount of hydrolysis of olive oil in one hour determined. To estimate the amount of

inactivation of enzyme which had taken place, the activity of the enzyme which had not been previously heated was determined in the usual manner.

Exp. 6. The mixtures incubated previous to determining the amount of hydrolysis contained

2 cc. buffer solution p_H 9·2 or 4·7
2 cc. solution containing 10 mg. $CaCl_2$
2 cc. solution containing enzyme
2 cc. amino-acid solution or water
2 cc. water.

Enzyme	p_H	Amino-acid added*	Hours of previous incubation	% hydrolysis: Previously incubated: Alone	% hydrolysis: Previously incubated: With amino-acid present	% hydrolysis: Freshly prepared
A. 0·5 cc. glycerol extract 4	9·2	Aspartic acid	1	13·9	18·0	19·2
B. ,, ,,	9·2	,,	21	8·1	11·1	19·2
C. 0·5 cc. glycerol extract 5	9·2	Histidine	4	11·4	14·0	14·7
D. ,, ,,	9·2	,,	24	0·5	3·3	14·7
E†. 1 cc. glycerol extract 4	9·2	Aspartic acid	4	5·3	18·6	31·4
F. 0·5 cc. glycerol extract 3	4·7	,,	4	5·2	5·2	7·3

* The final concentration of amino-acid was 0·01 *M*.

† No calcium chloride was added during the initial incubation.

A consideration of the above results shows that amino-acids increase the stability of lipase in alkaline but not in acid solutions. This is in agreement with the finding that amino-acids accelerate hydrolysis by lipase in alkaline but not in acid media.

It will be observed (Exp. 6, E) that the destruction of lipase in alkaline solution is more rapid when calcium salts are absent. This point is being investigated further, since it is of interest in connection with the claim by Willstätter, Waldschmidt-Leitz and Memmen [1923] that lipase from any source can be completely activated by the addition of calcium chloride, albumin and an ammonia-ammonium chloride buffer mixture. It has already been shown [Platt and Dawson, 1925] that the addition of small amounts of phosphates will further increase the activity of lipase in such a medium, and during the present investigation it was found that amino-acids can replace the albumin to produce a system which has a greater activating power for lipase than that of Willstätter.

Summary.

1. Amino-acids accelerate the hydrolysis, in alkaline or neutral but not in acid solutions, of ethyl butyrate and olive oil by pancreatic lipase.

2. The acceleration is probably due to the increased stability of the enzyme in alkaline solutions in the presence of amino-acids.

I wish to acknowledge my indebtedness to Prof. J. B. Cohen, in whose laboratory this investigation was commenced.

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LVIII. THE MICRO-DETERMINATION OF METALS IN SALTS.

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The practice of using salt formation as a means of purifying compounds of biological importance is one of the most commonly employed operations in biochemical studies, and it is therefore of the greatest importance to have an easy and certain method of analysing salts. The element which is generally selected for determination is the metal: and although the process of ashing or incinerating with sulphuric acid is so simple in theory the actual practical work is not only tedious but often leads to uncertain results. The cause of this must generally be ascribed to the persistent creeping of the sulphuric acid, and when, through lack or expense of material, it is necessary to do the analysis on a micro-scale, spitting or frothing also frequently adds to the difficulties.

Pregl has found it expedient to alter his original technique on several occasions [1924, 1]; and even with his present modification [1924, 2] the author and several others (private communications) have found it difficult or even impossible to obtain correct results with many salts, although others can be analysed with ease and accuracy. This uncertainty led the author to make some alterations in the method; and using the modifications described below he has been able to carry out many dozens of analyses on the most varied salts, always obtaining accurate results with certainty.

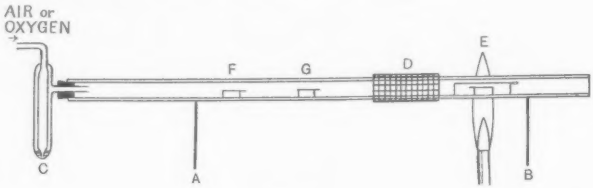

Fig. 1.

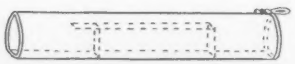

Fig. 2. (Full size.)

The chief modification consists in the use of a platinum cylinder[1] (Fig. 2) which surrounds the boat in which the combustion is done; any material which is generally lost by spitting or creeping is retained in the cylinder and finally weighed. The cylinder is about 40 mm. in length with a diameter of 8 mm. and is made of platinum foil 0·05 mm. in thickness. One part is flattened so that it can rest easily on a flat surface and the ends are strengthened with wire, one end also having a loop for ease of manipulation.

The cylinder and boat are boiled in dilute nitric acid (1 : 5), ignited, cooled on the block of the desiccator, placed side by side on the balance pan and weighed in the usual manner. Because of the comparatively large surface they should be allowed to remain five minutes on the pan so that they may assume a constant weight. 4 or 5 mg. of the substance to be analysed is weighed into the boat and is thoroughly moistened with two or three drops of sulphuric acid (1 : 5). It is of great advantage at this stage gently to evaporate off most of the water as this generally causes trouble in the incineration. For this purpose the boat is placed in the centre of the platinum cylinder which in turn is placed in a glass tube to protect it from dust. The whole is placed in a "regenerating" block at 120° for five minutes, a gentle current of air being drawn through the glass tube to remove the moisture.

The combustion is best carried out in a transparent silica tube (Fig. 1) about 45 cm. in length and 10 mm. internal diameter which is supported on an ordinary combustion stand *AB*. Pregl causes air to pass through the tube by using convection currents. This method has the obvious disabilities that the speed cannot be easily determined or controlled and therefore the author has found it much better to use an aspirator with a bubbler *C* to indicate the speed of the current. Moreover, when analysing salts with a high carbon content it is often difficult to burn all the carbon if only air is used and it is therefore advantageous also to have the tube connected with an aspirator containing oxygen.

After drying in the "regenerating" block the procedure of the combustion is to place the cylinder, which has the boat inside it, into the end of the silica tube, in the position shown in the figure. A fairly rapid stream of air is turned on and the flame slowly advanced from *D* to the centre of the cylinder *E*, using the wire gauze as shown to conduct the heat. This need not be done with the same great care as is necessary in the Pregl technique. After some time the gauze is removed and the full flame of the burner is allowed to play on the bare silica tube, the heat being also reflected by means of a semi-circular piece of coarse wire gauze placed over the silica tube. The air is gradually replaced by oxygen and after being heated strongly in this gas for three minutes the platinum cylinder may be removed to the desiccator, cooled and weighed as before. The course of the combustion can be watched quite well by looking through the end of the tube.

[1] The cylinder was made by Messrs Johnson, Matthey and Co., Ltd., Hatton Garden, London, for 28*s*., but the first experiments were done using a piece of foil rolled into a circular shape and this gave good results.

The author has never found any difficulty in obtaining the normal sulphates when using the above method and these give quite constant weights in air. It is obvious that if a deliquescent substance is obtained after incineration this can be weighed by placing the platinum cylinder in a suitable stoppered tube or weighing bottle after touching on the block of the desiccator. The author has also never found any reduction of the sulphates of the alkaline earths by carbon, but if there is any fear of this happening two or three drops of sulphuric acid can be distilled over the residue from small porcelain boats at *F* and *G*. In this way it is also possible to obtain excellent results from carbonates which have been obtained from ashing, etc. (cf. analyses of salt 4). Although the above experimental work has been given for incineration with sulphuric acid it is obvious that it can be used with great advantage for the generally simpler operation of ashing.

The accuracy of the method is shown by the analyses on the following pure substances (1–4). Dr H. W. Dudley was kind enough to send a few milligrams of the salts (5–8), the author only knowing the metal present. These were ordinary samples not specially purified.

No.	Salt and residue after ignition	Formula	Amount of salt taken mg.	Weight of residue mg.	Found %	Calc. %
1	Potassium tetroxalate	$C_4H_3O_8K$,	3·748	1·286	15·40	15·38
	(Potassium sulphate)	$2H_2O$	4·231	1·453	15·41	
2	Potassium nitrate	KNO_3	4·747	4·086	38·62	38·68
	(Potassium sulphate)		4·434	3·821	38·67	
3	Copper sulphate	$CuSO_4$,	4·414	1·412	25·56	25·46
	(Copper oxide)	$5H_2O$	4·747	1·521	25·60	
4	Lithium carbonate	Li_2CO_3	3·618	5·341	18·63	18·79
	(Lithium sulphate)		4·521	6·679	18·65	
5	Potassium hydrogen phthalate	$C_8H_5O_4K$	4·625	1·973	19·14	19·15
	(Potassium sulphate)		4·921	2·095	19·10	
6	Sodium succinate	$C_4H_4O_4Na_2$,	4·715	2·479	1·702	17·03
	(Sodium sulphate)	$6H_2O$	4·296	2·252	16·97	
7	Calcium oxalate	CaC_2O_4,	5·172	4·775	27·18	27·36
	(Calcium sulphate)	H_2O	4·424	4·104	27·30	
8	Barium benzenesulphonate	$C_{12}H_{10}O_6S_2Ba$,	3·778	1·884	29·35	29·25
	(Barium sulphate)	H_2O	3·487	1·733	29·25	

All the analyses on the above salts were carried out as unknowns for this communication by Mr A. Colwell of this laboratory and the author's best thanks are due to him.

The author wishes to take this opportunity of thanking Prof. Pregl for his hospitality to him. He also wishes to acknowledge a grant for apparatus from the Royal Society; the work was done during the tenure of a grant from the Medical Research Council.

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LIX. ON THE ALLEGED ACTION OF X-RAYS UPON CHOLESTEROL.

By IZRAEL HIEGER.

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(*Received March 7th, 1927.*)

The experiments described here are part of a study of the chemical changes produced in known constituents of cells by radiations of short wave-length. The first substance chosen for investigation was cholesterol, for the reason that it is present in practically all tissues, and changes are known to be produced in it by radiations of longer wave-length (ultra-violet light). It was hoped that the investigations would throw some light upon the changes in the tissues which lead up to X-ray cancer.

Experiments with X-rays.

In the greater number of experiments a solution of cholesterol was placed in a glass dish 5 cm. deep and 22 cm. in diameter which was then closed, to exclude nitrogen oxides present in the X-ray room, by parchment sealed down with adhesive strapping. The dish was placed 3 or 4 cm. below the unshielded X-ray tube; the whole of the dish then lay within the cone of rays. The tube used in these experiments was a deep therapy Coolidge tube with tungsten target, activated from a coil. The current through the tube was 4 m.a. The voltages indicated in Table I were estimated by means of a sphere-gap (10 cm. diameter spheres) and checked at intervals by the spectrograph. No filter was used. The distance from the anticathode to the surface of the solution was 14 cm. The dish and tube were kept cool by an air-fan, and the solutions were exposed to X-rays for times ranging from 30 to 90 minutes, with potentials of from 100,000 to 200,000 volts (Table I).

When a thin layer (*e.g.* 9 mm.) of a dilute solution of cholesterol in chloroform or carbon tetrachloride is exposed to penetrating X-rays (200 k.v.) for an hour, the residue on evaporation of the solvent is found to be no longer white and crystalline as in a control sample, but to consist of an amorphous gummy mass of greenish colour and of considerably lowered melting-point (50–100° as against 146° in the original cholesterol). At first sight this result seems to reveal a striking direct effect of the rays upon the cholesterol molecule, and this conclusion has actually been drawn by one observer [Roffo, 1925; see below]. But later experiments showed that the change observed in the cholesterol is secondary to decomposition brought about by the radiation in certain chlorine-containing solvents, namely chloroform and carbon

tetrachloride; if benzene be used as solvent no such change occurs (Table I), and again no change was observed when a gum-saline emulsion of cholesterol was irradiated.

Table I.

Action of X-rays upon solutions of cholesterol.

Solvent	Cholesterol grams in 100 cc.	Depth of solution mm.	Time of exposure minutes	Kilovolts *	Melting-point Irradiated ° C.	Melting-point Control ° C.	Lowering of melting-point °C.
Chloroform	0·02	170	60	200	125–130	143–145	16
,,	,,	,,	,,	100	140–143	141–144	1
,,	,,	120	,,	200	125–130	143–145	16
,,	,,	45	,,	200	100–115	141·5–145·5	35
,,	,,	,,	,,	100	137–141	144–146	6
,,	,,	9·5	,,	200	60–90	139–142·5	66
,,	0·05	,,	,,	,,	57–76	141–144	77
,,	0·075	,,	90	,,	60–72	141–144	77
,,	0·075	9	90	,,	65–71	143–145	76
,,	0·075	9	90	,,	59–65	—	c. 80
,,	0·05	4	30	,,	100–115	140–144	34
,,	0·044	,,	,,	175	107	—	c. 35
,,	0·044	,,	,,	,,	55	—	c. 87
Chloroform in completely filled tubes	0·06	,,	60	200	83–110	—	c. 45
Chloroform in current of N	0·06	5	60	200	106–113	—	c. 32
Carbon tetrachloride	0·02	170	60	200	124–131	144–145·5	17
,,	0·025	5·5	30	,,	70–90	140–143	62
,,	,,	4	30	,,	60–75		74
,,	0·05	4	60	,,	60–80	139–143	71
Monochlorobenzene	0·08	7	90	200	133–138	142–146	8
Benzene	0·02	6	60	200	143–144·5	143–145	0
,,	0·05	3	30	200	138–144	—	c. 2
,,	0·02	6	60	100	139–142	143–145	3
Gum-saline emulsion	0·02	9	90	200	138–142	—	c. 2

Action of radium upon solutions of cholesterol.

Chloroform	0·1	9	2 days	50 mg. $RaSO_4$ in Pt tube	140–143	145–146·5	4
,,	,,	,,	16 days	,,	125–133	144–146	16
,,	0·1	,,	16 days	40 mg. $RaSO_4$ in glass tube	50–70	140–144	82
Benzene	0·1	,,	2 days	50 mg. $RaSO_4$ in Pt tube	144–145	143–145	0
,,	,,	,,	18 days	,,	140–142	142–144	2

* The wave length of the shortest X-rays present is inversely proportional to the voltage used. 200 K.V. gives "hard" rays (*i.e.* more penetrating), 100 K.V. "soft" rays (*i.e.* less penetrating).

Details of technique.

All the solvents used were purified by drying and fractionation. The change in the cholesterol, in a suitable solvent, is increased by reducing the depth of the solution exposed; thus in chloroform the melting-point is reduced to 125–130° when the depth is 170 mm., and to 65–70° when the depth is 9 mm., the controls recovered from non-irradiated solutions in each case melting at 143–145°. It must be remembered, however, that the dose of rays received by the solution is considerably greater in a shallow vessel, as the

scattered rays from the bottom of the vessel then penetrate the whole depth of the solution. The minimum time of exposure necessary to produce any change was not investigated. It is evident that the effect depends upon the use of high voltages, for in comparable experiments (see Table I, chloroform) a potential of 200 K.V. gave a melting-point of 100–115°, while 100 K.V. gave a melting-point of 137–141°, which is but very little lower than that of the control (144–145°).

Roffo [1925] has described this conversion of cholesterol in chloroform solution by X-rays into an amorphous greenish material, but he makes the statement that this change occurs also when benzene is used as solvent, which is in complete contradiction to the observations reported in the present paper. Now the results given in Table I above show that no such change occurred in a benzene solution; the irradiated cholesterol was recovered with no change in melting-point (143–145° in the irradiated, and 143–145° in the control, sample). Further experiments showed that the amorphous product recovered from an irradiated solution in chloroform or carbon tetrachloride could not be a simple decomposition product of cholesterol[1]. The product was found to possess, in addition to the very low melting-point shown by many examples in Table I, the following properties: (1) a gain in weight of not less than 5 % over that of the cholesterol taken, (2) a chlorine content of from 3·8 to 4·9 % (estimations by Bacon's method [1909]). Although, in view of the great difficulty of removing the chlorine-containing solvent, caution is needed in drawing such a conclusion, there seems to be very little doubt that a chlorinated derivative of cholesterol is formed. As soon as the presence of chlorine in the product was detected, a sample of chloroform irradiated in an atmosphere of nitrogen was tested with potassium iodide and starch and was found to contain free chlorine. Hence the conditions required for chlorination are present[2]. The absence of change in a benzene solution, and in a watery emulsion, seems then to be due to the absence of organically combined chlorine. In order to investigate further the importance of a halogen element, a chlorine compound of a different type, namely monochlorobenzene, was used in one experiment as solvent. The lowering of melting-point was very slight (to 133–138° in comparison with 142–146°); hence the extensive change, giving melting-points from 60 to 100°, is not brought about by all organic chlorine compounds.

The possibility was considered that the effect was due to secondary radiations from the chlorine atom. To test this, a benzene solution of cholesterol

[1] In attempting to purify the gummy material, great difficulty was experienced in removing the last traces of solvent. It was found best to evaporate to apparent dryness *in vacuo*, then to redissolve in ether and again evaporate *in vacuo*, and to repeat this solution in ether and evaporation five times; it was hoped that in this way the ether would carry off the last traces of the less volatile solvent. In some experiments, which gave similar results, the removal of solvent was carried out *in vacuo* at 100°.

[2] The boiling-point of the chloroform used was found to be lowered by about 1° after irradiation. This is in disagreement with the results of Roffo.

was poured on to a layer of solid calcium chloride, and irradiated, but no significant change in melting-point was produced. The change is not dependent upon oxidation in a solution exposed to air, for the same lowering of melting-point is observed (1) when a chloroform solution is irradiated in completely filled corked tubes and (2) when a chloroform solution is saturated with hydrogen or nitrogen and exposed to a current of the gas throughout the period of irradiation (Table I).

Further evidence of the change brought about in cholesterol in chloroform solution is afforded by (1) a change in the rotatory power from $-46{\cdot}3°$ to $-21{\cdot}15°$ (green Hg line), (2) a diminution in the iodine value (Wijs) from 163, an abnormally high value such as is given by cholesterol, to 102.

Before the experiments upon the irradiation of chloroform alone had shown that the change in cholesterol was due to chlorination, experiments had been begun in which the amorphous product was tested upon animals for cancer-producing power. A solution in xylene of the material was painted twice weekly on the interscapular region of two mice, and on the ear of a rabbit, for 200 days, but no tumours were produced. Roffo and Correa [1924] have described a large decrease in the cholesterol content of tumours (rat sarcoma) exposed to X-rays. Any such decomposition of cholesterol *in vivo* can hardly be due to the process, dependent upon chlorination, which occurs in certain solvents *in vitro*. Experiments upon the cholesterol content of whole animals and of separate tissues before and after irradiation are now in progress in this Institute.

Experiments with radium.

A tube of glass or platinum 0·5 mm. thick containing radium sulphate was suspended in a glass tube containing about 20 cc. of cholesterol solution, so that a thickness of about 9 mm. of solution surrounded the tube. (For details, see Table I.) The cholesterol was recovered practically unaltered from solution in benzene. With a chloroform solution, radium in a platinum tube produced practically no change (140–143°) in two days, and a distinct change (125–133°) in 16 days; when a glass tube, which permits β-radiation in addition to γ-rays to pass, was used, a product exactly similar to that produced by X-rays at high voltage was obtained, namely an amorphous material melting at 50–70°. Radiations from radium have therefore the same action as have X-rays upon chlorine-containing solvents. Kailan [1917] had shown that chlorine is produced by the action of radium on chloroform and carbon tetrachloride, but I was not aware of his work when the experiments described here were carried out.

The decomposition of these chlorine compounds appears to depend upon rays of wave-length less than about 0·1 Å because (1) these would be produced by a voltage of 200 K.V. but not by 100 K.V.; as shown in Table I this voltage produces a large change in cholesterol (M.P. 55°) while with a voltage of 100,000 volts there is no change in the melting-point: and (2) the screening

action of the platinum tube is most effective upon the longer γ-rays, and the enclosure of radium in a platinum tube greatly diminishes the effect upon cholesterol (Table I).

Some months after the experiment reported in this paper had been completed, Dognon [1926] published some qualitative observations which confirm in every way the importance of the chemical nature of the solvent, and are in direct contradiction with the findings of Roffo. Dognon employed the Liebermann test as a criterion of change in irradiated solutions of cholesterol and found (1) that the test rapidly became negative when the solvent was carbon tetrachloride, chloroform, or bromoform, and (2) that no change occurred in benzene, carbon disulphide and monochlorobenzene. The additional negative result with another chlorine-free solvent, namely carbon disulphide, is a valuable confirmation of the dependence of the change upon a halogen.

Summary.

X-rays have been alleged to decompose cholesterol when in solution *in vitro* in various organic solvents, and it has been implied that similar changes occur *in vivo* when tumours are exposed to X-rays. The experiments described above show that X-rays act upon cholesterol only in certain chlorine-containing solvents (chloroform and carbon tetrachloride); in benzene no change is produced, and in monochlorobenzene the change is very slight. The decomposition of cholesterol in chloroform and carbon tetrachloride appears to depend upon a liberation of chlorine, the rays thus acting upon these solvents and not directly upon the cholesterol. Experiments with radium gave confirmatory results with regard to action on the solvent. In view of the dependence of the change upon chlorine-containing solvents it seems unlikely that this decomposition of cholesterol can occur in irradiated tissues.

Grateful acknowledgements are made to Dr J. E. A. Lynham of the Electrical and Radio-therapeutic Department of the Cancer Hospital for the very large number of hours which he has spent ungrudgingly in carrying out the numerous X-ray exposures. I wish to thank also Dr Leitch, the Director of this Institute, for allowing me the use of its facilities, and Dr Robert Knox, in whose Department the radiological work was carried out. This work was done during the tenure of a grant from the British Empire Cancer Campaign, to which body the author's best thanks are due, and was conducted under the supervision of Dr E. L. Kennaway, to whom he is greatly indebted.

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LX. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM. IV.

CARBOHYDRATE METABOLISM OF THE BRAIN TISSUE OF DEPANCREATISED CATS.

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From the Biochemical Laboratory, Cambridge.

(*Received February 28th, 1927.*)

It has recently been shown by Warburg, Posener and Negelein [1924], and by Loebel [1925], that brain tissue is able to break down glucose to lactic acid with great rapidity.

We found that the lactic acid content of the excised brain (rabbit) did not rise appreciably above the "resting" level on incubation of the tissue under anaerobic conditions, although large amounts of lactic acid were formed when glucose was supplied to the tissue [Holmes and Holmes, 1925, 1]. It seemed, therefore, that the failure of the excised brain to make lactic acid must be due to a lack of glucose in the tissue.

A certain amount of reducing substance has been found in the tissue, which we have shown [Holmes and Holmes, 1926, 1] to consist mainly of creatine and creatinine, with about 30 mg. % pentose. There is no evidence for the presence of any glucose. There is a small amount of glycogen in the brain, but it does not give rise to any lactic acid, and is not lessened in amount upon incubation, whether aerobically or anaerobically, at alkaline p_H [Holmes and Holmes, 1926, 2]. E. G. H. has lately been able to show that the glycogen content of the brain tissue does not fall after depancreatisation, when the glycogen in the body tends to disappear, nor after a convulsant dose of insulin, although the brain is then suffering from a shortage of glucose. It appears, therefore, that the glycogen of the brain is in some way immobilised, and does not play the part one would expect in the carbohydrate metabolism of the brain tissue.

From consideration of these various facts we concluded that the brain contained little or no lactic acid precursor. On the other hand, it contains a by no means negligible (about 100 mg. %) amount of lactic acid after death, and the most likely origin of this lactic acid is the glucose of the blood. Though some of this glucose is probably present in the brain during life, it is all converted into lactic acid in the time (about $3\frac{1}{2}$ minutes) elapsing between the death of the animal and the removal and rapid cooling of the brain.

If this is indeed the fact, one might expect the lactic acid content of the brain to be dependent upon the level of glucose in the blood. We have been able to show that this is apparently the case, and thus to support our view of the origin of the lactic acid in the brain.

The experiments involved are described below. The method employed for the estimation of the lactic acid is in no essential different from that of Meyerhof [1920]. Rabbits were used and the blood was obtained from an ear vein immediately before the death of the animal, which was killed by a blow on the neck. The results of the experiments are shown in Table I.

It will be seen from Table I that as the level of the blood sugar falls after the administration of insulin to the rabbit, the brain lactic acid content falls in a corresponding manner. When the hypoglycaemia is severe, the amount of lactic acid in the brain is very small. These results, which indicate that a convulsant dose of insulin produces considerable glucose deprivation in the central nervous system, are described in earlier papers [Holmes and Holmes 1925, 1, 2]. It is well to emphasise the point once more, that under these conditions of carbohydrate deprivation, the glycogen in the brain remains at a normal level. For this reason, too, the decrease in lactic acid content of the brain cannot be due to a local increase of glycogen formation in that organ, as the following figures show—a starved rabbit, just about to convulse after a dose of insulin, showed a brain lactic acid content of 36·5 mg. % and a glycogen content of 25·5 mg. %. The normal lactic acid content is 90–110 mg. %, so that, to account for the drop in lactic acid, the brain would have to contain at least 55 mg. % of extra glycogen, in addition to any which might have been present before the insulin was given.

Table I.

Exp.		Blood sugar mg. %	Brain lactic acid mg. %
1	Killed with chloroform	273	163
2	„ „ ether	260	159
3	Intravenous injection glucose	244	153
4	„ „ „	217	146
5	„ „ „ 11 mins. previously	208	113
6	Anaesthetised with urethane	191	134
8	Intravenous glucose 27 mins. previously	183	123
9	Normal	160	97·4
10	„	150	101
11	Insulin	124	96
12	Amytal	123	85
13	Insulin. No symptoms	107	88
14	„ No symptoms	95	76
15	„ Flaccid	65	49
16	„ Commencement of convulsion	50	45
17	Insulin convulsions well marked	—	29
18	Convulsed with insulin: recovered with glucose ...	119	56

The converse of this effect of hypoglycaemia can be seen when the blood sugar level is raised by any means. After the injection of glucose, for instance, the brain lactic acid level is high, as it is after the administration of adrenaline, or after killing with chloroform or ether, all of which processes raise the

blood glucose level. Table I gives some typical examples, showing the correspondence between the blood sugar and brain lactic acid levels.

Gesell [1925] traces a direct relationship between the level of the lactic acid in the brain, and the activity of the respiratory centre. Our experiments do not support this view, since the hyperglycaemic animals showed no hyperpnoea, while animals on the verge of insulin convulsions pant heavily.

It is interesting to notice that in deep anaesthesia the relationship between brain lactic acid and blood sugar still persists. Chloroform, ether, and urethane are known to exert a much greater inhibitory effect on the oxidative processes of brain than upon glycolysis [Loebel, 1925]. The rise in lactic acid might, therefore, be in part due to a failure in removal rather than to an increased production. The figures, however, do not suggest this, falling as they do into series with the others, nor have we been able to find any increase in cases of light amytal anaesthesia, which drug, in moderate doses at least, does not raise the blood sugar.

When the blood sugar has been abruptly raised by the injection of glucose, there is, of course, some delay before the brain lactic acid is raised correspondingly. This, doubtless, accounts for some of the irregularities in brain lactic acid level observed after the intravenous injection of glucose in normal animals, and also for the fact that in these animals the brain lactic acid level does not rise as high as it does in the case of depancreatised animals with the same blood sugar level. Exp. 18 of Table I illustrates this point, the animal having been killed a few minutes after the injection of the glucose.

Since the formation of lactic acid is known to be necessary for the process of muscular contraction, it has long been puzzling to note that there is no marked failure of this process in extreme diabetes. The problem which confronted us was a somewhat similar one. The fact that an animal will fall into convulsions when its brain is deprived of lactic acid precursor by the lowering of the blood sugar after insulin, and will recover from these convulsions when the supply of glucose is restored, indicates that the central nervous system is dependent for some purposes upon its carbohydrate metabolism. Yet, except indirectly through ketosis, there are no marked symptoms of any impairment of the functions of the central nervous system in diabetes. This was very plainly apparent in the case of our depancreatised cats, which showed no ketosis, and remained active and intelligent.

Recent work [Himwich, Loebel and Barr, 1924; Hetzel and Long, 1926] has shown that the formation of lactic acid takes place in diabetic as in normal muscles, and it is simple to show that lactic acid is present in the brain of the depancreatised animal. But whereas it is extremely difficult to determine directly the origin and fate of the lactic acid in the diabetic muscle, it should be far easier to determine the origin, at least, of the lactic acid in an organ like the brain, which has little or no precursor in its own tissues, and is dependent upon an outside supply.

We have considered the possibility that the amount of lactic acid in the

brain may depend upon the level of the lactic acid in the blood, that is, that its presence may be due in part to a passive accumulation from the blood. The figures of Himwich, Loebel and Barr [1924] do not indicate that any rise in the lactic acid level of the blood occurs as a result of diabetes, except after exercise, so that we do not need to give particular consideration to this point in connection with our depancreatised animals. We have carried out a few experiments comparing the blood lactic acid and the brain lactic acid levels, which show that, as far as the experiments on rabbits are concerned, there is no reason to suppose that the changes in the lactic acid content of the brain are due to changes in the lactic acid content of the blood.

Owing to the extreme difficulty of depancreatising rabbits, it was decided to use cats for the following experiments. We had had no previous experience with cats, and therefore thought it necessary to carry out some determinations of the blood sugar and brain lactic acid levels in normal animals. The results (Table II) show that, in cats as in rabbits, the brain lactic acid level depends upon the blood sugar level. The normal lactic acid values are fairly constant. In two cases only (one normal and one diabetic) was there any increase in the lactic acid content of the brain after incubation; in both of these the brain had been badly injured by the blow which killed the animal. The glycogen of the brain showed no corresponding fall, and we had to assume that the breakdown of the glucose remaining in the brain had in some way been retarded. These cases supported our view that a detectable amount of the glucose entering the brain from the blood is present during life, but is usually broken down directly after death. For comparison with the other animals it seemed best to take the higher value obtained in each of these two cases, and when this was done the results agreed well with those obtained in other experiments.

Both the normal and the depancreatised animals were killed with one heavy blow on the head, death being instantaneous. The brain was removed, and was then cut in halves and one half frozen immediately, or it was quickly chopped and sampled and a portion frozen. The latter method gave the best sampling, and the slightly increased delay before freezing made no difference to the level of the "resting" values obtained. The frozen portions were weighed out and immersed in alcohol whilst still very cold, and were used for the estimations of the "resting" lactic acid content.

The estimations were carried out by the method of Meyerhof, with the following very slight modification. Since the greasiness of the tissue makes filtration through paper impossible, the alcoholic extracts were all filtered through Jena glass filters (porosity < 7 or 5–7).

For experiments dealing with the effect of aeration or of anaerobic incubation upon the lactic acid content of the tissue, the portions of the brain which had not been subjected to cooling were employed.

As we wished the depancreatised animals to be as normal as possible in every other respect, they were given injections of insulin, and sometimes of

glucose also, for the first few days after the operation[1], this treatment facilitating recovery from the anaesthetic and healing of the scar. Cat 5, which was pregnant, and Cat 4 (Table II), refused to eat and were therefore killed three and five days, respectively, after the operation. Two out of the four animals operated on recovered very well (Cats 3 and 7, Table II) and these were killed eight and fourteen days respectively after the operation. At the time of death they appeared to be in good health, took a good deal of exercise, ate enormously, and were perfectly intelligent. Cat 7 excreted 5 g. of glucose per 100 cc. urine the day before its death, although it was fed entirely on lean meat with a little diluted milk. In no case could we detect any ketone bodies, by Rothera's test, in the urine of the animals, and the fact that the diabetes was uncomplicated by ketosis probably accounted in part for the surprisingly good health they displayed.

After death all the animals were very carefully examined for possible residual portions of pancreas, and any suspected fragments of tissue were taken for section. In no case, however, was any pancreatic tissue found.

As we could detect no acetone or acetoacetic acid either in the urine of the depancreatised animals or in the extracts of the brain tissue in which the lactic acid was to be estimated, we made no allowance for these substances in our estimations.

The estimations by Meyerhof's method showed the presence of a considerable amount of lactic acid, and the thiophene test for lactic acid was given by the solutions.

If this lactic acid arises from the glucose of the blood as we had found to be the case in normal animals, and if the power of the brain tissue to break down this glucose is not decreased after depancreatisation, then the greatly increased blood glucose level found in diabetic animals should lead to an increased production of lactic acid by the brain. This we did, in fact, find to be the case (see Table II).

Table II.

	Blood sugar mg. %	Brain lactic acid mg. %
Cat 5. Diabetic. Killed 3 days after depancreatisation. Pregnant; anuric for 24 hours	440	220
Cat 7. Diabetic. Killed 14 days after depancreatisation. Insulin for first 9 days. Subcutaneous injection of glucose 25 minutes before killing. Appeared healthy	317	200
Cat 4. Diabetic. Killed 5 days after depancreatisation. Would not eat. Given glucose and insulin for first 3 days. Subcutaneous injection of glucose 45 minutes before killing	311	187·5
Cat 3. Diabetic. Killed 8 days after depancreatisation. Had insulin for first 3 days. Appeared healthy	245	170
Cat 1. Killed with ether. Blood sugar certainly raised	—	127
Cat 7. Normal	123	104
Cat 8. Normal	—	90
Cat 9. Normal	110	110·5
Cat 2. Insulin. Killed in flaccid stage	—	57

[1] We wish to express our gratitude to Dr H. H. Dale for instructing us in the technique of this operation, and for allowing us to witness a depancreatisation which he himself performed upon a cat.

It will be seen that the brain lactic acid values correspond quite well with those obtained in normal animals when the blood sugar is artificially raised.

Thus, so far as we can judge from the data in Table II the brain tissue of a completely depancreatised cat is still capable of converting the glucose of the blood into lactic acid. A high level of glucose in the blood produces a high level of lactic acid in the brain of depancreatised as well as of normal animals. It is therefore difficult to imagine that the lactic acid which is present in the brain tissue of the depancreatised animals is derived from some unknown and abnormal source. Moreover, the excised brain tissue, taken from a completely depancreatised animal (Cat 3, 8 days after operation) was found to be capable of breaking down large amounts of glucose to lactic acid *in vitro*. The tissue was chopped and placed in Ringer's solution containing 0·25 % glucose, and potassium cyanide was added (final strength $M/650$) to prevent oxidation of the lactic acid. After standing for 24 hours at room temperature, the amount of lactic acid present had increased from 171 mg. % (the "resting" value) to 365 mg. %. Tissue kept under similar conditions, but without added glucose, showed no increase in the amount of lactic acid present.

We feel safe in assuming that in all probability the origin of the lactic acid in the brain of diabetic animals is the same as that in the brain of normal animals. Of the subsequent fate of the lactic acid we know nothing, except that it is removed by some oxidative mechanism when the excised tissue is well aerated, as one would expect from Warburg's work [Warburg, Posener and Negelein, 1924], and that the power to effect this oxidative removal is retained by diabetic tissue.

The figures (Table III) showing the disappearance of lactic acid from normal and depancreatised brain tissue, illustrate this point, and also show the activity of brain tissue in oxidising preformed lactic acid. The conditions of the experiments were as follows.

The brain was chopped and weighed. One portion of it was then used immediately for the determination of the "resting" level of the lactic acid; the other portion was placed in a stoppered bottle with about 10 cc. of Ringer's solution (p_H 7·4) and shaken in a bath at 37°, to ensure thorough aeration for about 3 hours.

Table III. *Lactic acid values expressed in mg. per* 100 *g. tissue.*

	"Resting" lactic acid mg.	Lactic acid after aeration mg.	Decrease mg.
Rabbit. Normal	91	15	76
Rabbit. Normal	97	36	61
Cat 6. Normal	104	41	63
Cat 4. Depancreatised	187	92	95
Cat 7. Depancreatised	202	111	91

It can be seen from this table that the power to remove lactic acid by oxidation is in no way decreased after depancreatisation.

SUMMARY.

1. The apparent absence of preformed lactic acid precursor from the brain, and the stability of the glycogen store in that organ, are discussed, and evidence is brought forward showing that the lactic acid found in the brain is formed from the glucose supplied by the blood. The lactic acid values fall and rise with the blood sugar, both in hypo- and hyper-glycaemic conditions.

2. Hyperglycaemia due to the administration of anaesthetics is accompanied by increased brain lactic acid values.

3. The brains of completely depancreatised cats show a resting lactic acid value corresponding to the degree of hyperglycaemia existing at the time of death.

4. The brain tissue of diabetic, like that of normal animals, is capable of converting glucose to lactic acid, and of removing lactic acid under aerobic conditions.

One of us [B. E. H.] is in receipt of a grant from the Medical Research Council.

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LXI. A STUDY OF THE EFFECT OF HEAT AND OXIDATION ON COD-LIVER OIL AS MEASURED BY COLOUR TESTS.

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(*Received March 4th, 1927.*)

In a previous communication [Willimott, Moore and Wokes, 1926], the destruction of vitamin A in cod-liver oil by concentrated sulphuric acid, by phosphorus pentoxide and by ultra-violet light was followed by means of the four principal colour tests put forward for the detection of this vitamin, viz.: concentrated sulphuric acid [Drummond and Watson, 1922]; powdered phosphorus pentoxide [Fearon, 1925], arsenic trichloride [Rosenheim and Drummond, 1925] and antimony trichloride [Carr and Price, 1926].

It was found that while these reagents all gave comparable results, the last two were much more sensitive and were in agreement when applied quantitatively, a Lovibond tintometer being used to measure the intensity of blue colour, which is now regarded by many as an index of the vitamin content [Rosenheim and Drummond, 1925; Takahashi *et al.*, 1925]. It was also attempted to follow the destruction of the vitamin when the oil was aerated at 100°, but a difficulty was encountered in the darkening in colour of the oil produced by heating which interfered with the colour tests. This difficulty has since been overcome, and a detailed study has been made of the effect of aeration at different temperatures.

Most of the previous work has been carried out on butter fat as the source of vitamin A, and conflicting results have been obtained. It seems probable that these discrepancies have been increased by the fact of the wide variation in vitamin content of different samples of butter and by the fact that the fat-soluble vitamins had not then been differentiated. With the exception of Osborne and Mendel's studies [1915, 1920], the earlier work in America [Steenbock, Boutwell and Kent, 1918] and also in this country [Drummond, 1919] had suggested that the destruction of vitamin A was due to heat alone and not to oxidation. Hopkins' investigations, however, carried out in 1919 [1920, 1] gave the first clear explanation when he showed conclusively that aeration as well as heat was an essential factor in the destruction of vitamin A in butter fat [1920, 2].

Hopkins [1920, 2] found that after one hour's aeration at 120° the butter still maintained a steady though subnormal growth throughout the experimental period (11 weeks), and that even after 2 hours' aeration the vitamin

was not completely destroyed. Drummond and Coward [1920] found 3 hours' aeration at 96° sufficient for complete destruction and if this process is a chemical one, a rise of 24° might be expected to reduce the time below 2 hours. Moreover, Rosenheim and Drummond [1925] found that one hour's aeration of cod-liver oil at 100° completely destroyed its vitamin A. In view of these conflicting results we decided to investigate the effect of aeration at different temperatures above and below 100°, using colour tests to measure the content of vitamin A. Although the specificity of the latter is not yet conclusively established, our experience leads us to think that, by the adoption of certain precautions, it is possible to make them reliable with a much smaller experimental error than is the case with animal experiments. We have also found that, in any given sample of oil, partial destruction of the vitamin either by irradiation [Willimott, Moore and Wokes, 1926; Willimott and Wokes, 1927] or by aeration (unpublished results) appears to render the remainder of vitamin A present in the oil unstable, so that it continues decomposing, and nearly half may be lost during the time required to carry out an animal experiment. For these reasons we have relied mainly upon colour tests.

Experimental.

A sample of Norwegian cod-liver oil, known by feeding experiments to be potent in vitamin A, was used in this investigation. About 50 cc. were placed in a boiling-tube and heated in a water-bath until the temperature of the oil became steady at 97–98°. A brisk current of air was then drawn through at the rate of 4 to 6 bubbles per second. At the end of every 5 minutes after aeration started, a few cc. of the oil were withdrawn by means of a pipette, and transferred to tightly-stoppered bottles of non-actinic glass. During withdrawal of the samples the air current was discontinued for 15 to 20 seconds. These samples of oil which had been aerated at 98° for known periods of time were at once examined for their content of vitamin A by the four colour tests. The results, which are given in Table I, show that under the conditions of our experiment, the vitamin A in cod-liver oil is destroyed by about 75 minutes' aeration at 98°. It will be noticed that, as in our previous work on the destruction of vitamin A by irradiation, concentrated sulphuric acid and phosphorus pentoxide were found less sensitive reagents than arsenic trichloride and antimony trichloride. With these last two reagents, the initial blue colour fades gradually to green, the time taken decreasing as more of the vitamin is destroyed. The end-point was taken as the time when the change from blue to green was practically instantaneous, and was probably correct to within 5 minutes.

We next attempted to measure the vitamin content of each of these samples of aerated cod-liver oil by means of the arsenic trichloride and antimony trichloride reagents, using a Lovibond tintometer. Since publishing our previous communication [Willimott, Moore and Wokes, 1926], we have

made a thorough study of the antimony trichloride test for vitamin A, and reached further conclusions of some importance, which may be summarised as follows [Wokes and Willimott, 1927].

Table I. *Colour tests on cod-liver oil which has been aerated at 98° for different periods.*

Time aerated (minutes)	Conc. H_2SO_4	P_2O_5	$AsCl_3$	$SbCl_3$	Starch iodide test
0	+ +	+ +	+ +	+ +	−
5	+ +	+ +	+ +	+ +	+
10	+	+	+ +	+ +	+
15	+	+	+ +	+ +	+
20	+	+	+ +	+ +	+
25	+	+	+ +	+ +	+ +
30	±	±	+	+	+ +
35	−	−	+	+	+ +
40	−	−	+	+	+ +
45	−	−	+	+	+ +
50	−	−	+	+	+ + +
55	−	−	+	+	+ + +
60	−	−	+	+	+ + +
65	−	−	+	+	+ + +
70	−	−	±	±	+ + + +
75	−	−	−	−	+ + + +

NOTE. The starch iodide test was applied by putting a drop of aerated oil on a piece of filter paper previously soaked in an aqueous solution of soluble starch and potassium iodide (about 1 % of each) and then dried. If a yellow colour could be observed within 5 seconds, the test was considered to be + + + +, between ¼ and 1 minute + + +, between 2 and 5 minutes + +, more than 10 minutes +.

The reaction consists of a series of colour changes—blue—yellow—red, with intermediate shades. It can be retarded by using anhydrous solvents, and working at a low temperature. In practice 15° or 16° appears to be the most suitable temperature, and it is suggested that correction be made for any wide deviation from that figure. (The temperature coefficient of the reaction is approximately 2.) The reaction is accelerated when the vitamin is rendered unstable by irradiation or aeration. The tintometer reading should be taken exactly 30 seconds after mixing, and as the colour is continually changing, the changes originating at the top of the liquid where there is exposure to air, it is necessary to mix the liquid continually with a small glass rod to ensure a uniform blend of colour. When about two-thirds of the vitamin has been destroyed, the blue colour has practically all disappeared within 30 seconds after mixing, and it is therefore impossible to obtain a tintometer reading, although the colour tests are still quite definitely positive.

Destruction of the vitamin appears to produce substances which give an *immediate* yellow colour with the reagent. We find that when working with irradiated or aerated oils it is necessary to put in more yellow units for the initial reading in order to secure an accurate colour match at the 30 second interval. If, however, a test is made on a sample of oil in which the vitamin has been completely destroyed, the colour immediately produced is red-brown. This same colour is finally reached in any test, although in a stable oil it may not be attained for over an hour.

When the concentration of cod-liver oil in the reaction mixture is plotted against blue colour produced by either arsenic trichloride or antimony trichloride, we find in the case of all the oils so far examined that the results cease to be a linear function above a concentration of 2 to 3 %. We therefore suggest that the amounts taken be arranged so that the readings of Lovibond blue units, using a half-inch cell, are not higher than 15 to 20.

The experimental findings which we have described above show the necessity of adopting various precautions when using either arsenic trichloride or antimony trichloride as reagent for vitamin A. We have now carried out some hundreds of observations on oils from Norway, Newfoundland and Iceland, and have formed the opinion that, when these precautions are observed, the experimental error on the results obtained is probably less than 10 %.

We next estimated by means of the antimony trichloride reagent the vitamin A content of each of the samples of cod-liver oil which had been aerated at 98° for different periods of time, taking in each case 0·2 cc. of a 20 % V/V solution of the oil, and mixing in a half-inch cell with 2 cc. of a solution of antimony trichloride in anhydrous chloroform, about 26 % W/V. In doing so we adopted all the precautions suggested by our previous experience. At least three readings were taken on each sample, and found to agree within 10 %. A number of readings were also obtained with arsenic trichloride, using the technique previously described [Willimott, Moore and Wokes, 1926], and were found to agree with those obtained with antimony trichloride within the limits of experimental error. The mean results are plotted in Fig. 1, in which the curve marked 98° represents the effect on vitamin A, as measured by colour tests, of aeration at 98° for different periods of time, under the conditions of our experiment. The temperature of the oil was taken every minute, and was found not to vary more than a degree either way, with an average value of 98°. The rate of aeration we had adopted was approximately the same as that of Hopkins [1920, 2]. In order to test the effect of increasing the rate of aeration, a second experiment was carried out in which the air was drawn through at a maximum speed of 10 to 12 bubbles per second (practically a continuous stream), the temperature being maintained at 98°, and results estimated as before. The results of these two experiments showed the variation to be less than the experimental error.

Further experiments were then carried out at 88°, 108°, 118°, and 125°, using a paraffin-bath in the last three cases, and adopting the same precautions. The results are plotted in continuous lines in Fig. 1. In order to estimate how much of the destruction of the vitamin was due to heat *per se*, in each experiment a blank was run by putting in the bath a test-tube of oil loosely plugged with cotton-wool, from which samples were withdrawn at 15 minute intervals. The vitamin content of each of these samples was also estimated in the same manner, and the results for each temperature are shown by the broken lines.

Turning to the effect of aeration on vitamin A, we found that the time required for complete destruction was 105 minutes at 88°, 75 minutes at 98°,

50 minutes at 108°, 35 minutes at 118°, and 30 minutes at 125°. These results agree closely with those obtained by previous workers, using both animal experiments and colour tests.

The temperature coefficient for the destruction of vitamin A by aeration, calculated from our data for each 10 degrees rise between 88° and 125°, would seem to be between 1·4 and 1·5. This is similar to the temperature coefficient of 1·5 obtained by Delf [1918] for the destruction of vitamin C by heat.

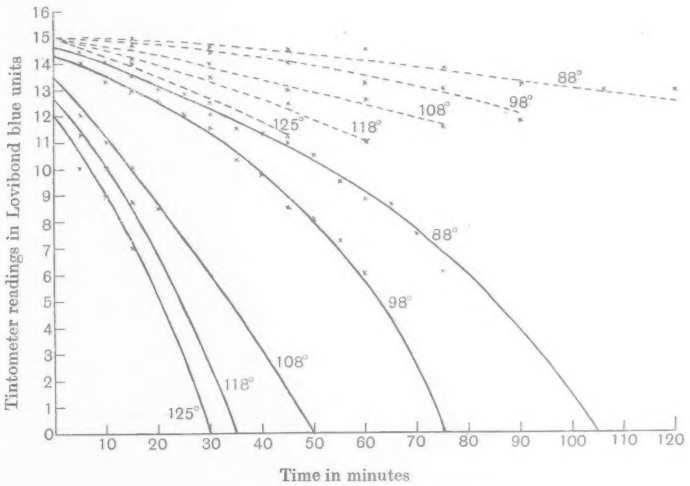

Fig. 1. Effect of heat and oxidation on vitamin A.

Similar results were obtained in the case of the oil heated without aeration at the same series of temperatures. Since there was contact with air at the small surface in the tube, the rate of destruction, although much less than that of the vitamin in the oil aerated at the same temperature, was greater than has been observed by previous workers in the case where air was strictly excluded.

In view of the opinion that the destruction of the vitamin is due to the oxygen of the air, we thought it of interest to examine the aerated oils for oxidising substances. Zilva [1920, 1922] has shown that the destructive action of ultra-violet rays is due to the ozone which they have produced in the surrounding air. Rosenheim and Webster [1926] obtained evidence of the presence of peroxides in cod-liver oil in which the vitamin A had been destroyed by exposure to air and sunlight at room temperature. In our own experiments on the destruction of vitamin A in cod-liver oil by ultra-violet light, we have been able to detect ozone in the samples of irradiated oils, after removal from the vicinity of the mercury vapour lamp, in amounts which

steadily increased as the destructive action proceeded. In the case of the destruction of the vitamin by aeration, we do not think that ozone is an active agent. Schönbein [1858] certainly suggested that ozone may be produced in the slow oxidation of various oils containing unsaturated hydrocarbons, but this suggestion was refuted by Engler [1898, 1904] who stated that the tests obtained were due, not to free ozone, but to unstable peroxides. For instance, turpentine after shaking with air will colour starch iodide paper blue, decolorise methylene blue, etc. We therefore applied, both to the aerated oils immediately after collection, and to the air which had been drawn through them, a number of tests considered characteristic of ozone, of hydrogen peroxide, and of organic and other peroxides. The results, which are summarised in Table II, would seem to indicate that the destruction of vitamin A by aeration is probably due to volatile organic peroxides. This tentative conclusion is of interest in connection with our previous suggestion [Willimott and Wokes, 1926, 2] that the distribution of vitamin A in the *Citrus* fruits may possibly be related to the presence of peroxidases.

Table II. *Tests for oxidising substances in cod-liver oil after aeration.*

Test applied to	Starch iodide	Acid $KMnO_4$	Tetramethyl base	$Ti(SO_4)_2$	Guaiacum and peroxidase
Oil before aeration	−	−	−	−	−
Oil after aeration	+	+	−	−	?
Air before passage through aerated oil	−	−	−	−	−
Air after passage through aerated oil	+	+	−	−	?
Ozone	+	−	+	−	−
Hydrogen peroxide	+	+	−	+	+
Organic peroxides	+	+	−	−	±

NOTES.

When the air, after passage through the oil, was bubbled through strong chromic acid before passing into starch iodide, a negative result was obtained. This method is suggested by Mellor [1922] for differentiating between ozone (gives positive) and peroxide (gives negative).

As a source of peroxidase free from oxygenase we used an aqueous extract of the colourless residue obtained by exhausting the rind of lemons with 90 % alcohol, according to the method of Onslow [1919]. We have previously shown this to contain peroxidase only [Willimott and Wokes, 1926, 1]. The tincture of guaiacum had been boiled with charcoal to remove peroxides.

As sources of organic peroxides we used various autoxidised oils, such as turpentine and orange rind oil, heated to 98°.

SUMMARY.

Cod-liver oil was aerated at different temperatures between 88° and 125°, and colour tests were made at frequent intervals for vitamin A. Qualitative agreement was obtained with the four tests, concentrated sulphuric acid, phosphorus pentoxide, arsenic trichloride and antimony trichloride, and the last two gave the same end-point for complete destruction of the vitamin.

Arsenic trichloride and antimony trichloride were then applied quantitatively to estimate the vitamin. Curves were obtained representing the course of destruction of the vitamin at 88°, 98°, 108°, 118° and 125°. The temperature coefficient for 10° was found to be about 1·4 to 1·5. These results were in

agreement with observations made by other workers, using animal experiments.

The results of a preliminary series of tests applied to the oils after aeration indicated that the destruction of the vitamin may have been due to volatile organic peroxides.

We are indebted to Professor E. C. C. Baly for the loan of the tintometer.

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LXII. A NOTE ON THE AMMONIUM SULPHATE PRECIPITATION OF THE ACTIVE PRINCIPLE OF THE CULTURE FILTRATES OF *C. DIPHTHERIAE*.

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(*Received March 1st, 1927.*)

In previous papers [Watson and Wallace, 1924, 1, 2; Watson and Langstaff, 1926] the methods of preparation and some properties of the acid-precipitable specific principle of culture filtrates of the diphtheria bacillus have been described. The investigation has been extended to the examination of the precipitates produced by various concentrations of ammonium sulphate, the activity of the precipitates being again estimated by the *in vitro* flocculation test of Ramon [1922]. Throughout the paper the term "active principle" has been used to denote the substance (or substances) present in culture filtrates of *C. diphtheriae* which gives this specific *in vitro* flocculation with antitoxin. While it is probable that these flocculation values are by themselves rarely a reliable index of the antigenic value of the solutions, they obviously afford a valuable method of estimating the combining power of solutions obtained by chemical methods of purification of culture filtrates.

Heineman [1908] utilised the ammonium sulphate method for concentrating diphtheria toxin, and in one case obtained a solution ten times as strong as the original filtrate. Some loss of specific principle—as measured by animal tests (guinea-pig M.L.D.)—always occurred. Seibert and Long [1926], in a recent investigation of the chemical composition of the active principle of tuberculin, used glacial acetic acid and ammonium sulphate as precipitants. While the precipitation was always incomplete with the former, complete saturation with ammonium sulphate appeared to precipitate the whole of the tuberculin activity and the authors advanced experimental evidence to show that the active principle was a whole protein.

Experimental.

The solutions of the crude active material were prepared, except where otherwise stated, by inoculating media made by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927] with P.W. 8 strain of *C. diphtheriae* and growing for ten days in the incubator. The cultures were then treated with 0·5 % toluene, 0·5 % phenol or 0·75 % of neutralised formalin

(40 % formaldehyde), filtered after 24 hours and tested for Lf value[1]. The culture filtrates were grouped according to Lf values.

For the removal of ammonium sulphate or acid-precipitable material small volumes were filtered through paper. Where large volumes were being dealt with the precipitates were recovered quantitatively by centrifugation in a Sharples' centrifuge, the bowl of which was provided with a detachable celluloid roll, although it is of interest to note that Siebert and Long found the high specific gravity of their ammonium sulphate-tuberculin mixtures (containing glycerol) precluded this. The acid-precipitable material was dissolved in caustic soda to p_H 8·0 and the sulphate-precipitable material in distilled water, in each case to the original volume or to an aliquot part. The very small amount of ammonium sulphate present in these purified solutions seemed to interfere in no way with the flocculation test with antitoxin, although it was difficult to test solutions containing more than traces of the salt owing to rapid precipitation of the serum proteins. One of the features of the testing of the more concentrated solutions was the extreme rapidity of the flocculations. Very active concentrated solutions frequently flocculate in a few minutes at 37°. On diluting these solutions the time of flocculation gradually increases, and this time is not materially altered whether the diluent is water, tryptic digest broth, muscle extract or a two per cent. solution of peptone. The organic nitrogen of the purified solutions containing small amounts of ammonium sulphate was estimated after removing the ammonia with potassium bicarbonate [see Haslam, 1905].

1. *The precipitation of the active principle at various levels of ammonium sulphate concentration.*

To equal volumes of culture filtrates which had been treated with formalin, readjusted to p_H 8·0, filtered through a Berkefeld candle and incubated for a week at 36°, varying amounts of ammonium sulphate were added and the precipitates produced at the complete, two-thirds, one-half and one-third saturation levels were examined. The mixtures were allowed to stand overnight before filtration. The precipitates were washed with ammonium sulphate solution at the corresponding level and then dissolved in water. Estimations of the organic nitrogen and the specific activity of the solutions were made.

The filtrates from these initial precipitations were brought up to complete saturation by addition of solid ammonium sulphate and the second precipitates were washed, collected, dissolved in water, and tested as before.

It will be seen from Table I that the maximum recovery of the active principle is effected by complete saturation with ammonium sulphate, although similar recoveries can be made by precipitation in two successive

[1] As used in this paper the Lf dose may be defined as that volume of any solution containing the active principle of the culture filtrates of *C. diphtheriae* which is equal to one unit of antitoxin by the flocculation test (for fuller definition and description of the test see Glenny and Okell [1924] and Glenny and Wallace [1925]).

stages as shown. For the toxoid under consideration, however, the most pure active precipitate was obtained by two-thirds saturation—the precipitate containing 162 Lf units per mg. of nitrogen. The original toxoid contained 4 Lf units per mg. of nitrogen, so that a relative nitrogen purification of over 40 was effected.

Table I. *Precipitation of a diphtheria toxoid at various levels of saturation with ammonium sulphate.*

Original toxoid. TME 146 containing 14,000 Lf units per l. and 4 Lf units per mg. N.

	Initial recovery of Lf units				Recovery of Lf units by completing saturation of filtrates from first precipitate			
Degree of saturation	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.
Complete	11,700	84	248	47	—	—	—	—
$\frac{2}{3}$	11,500	82	71	162	200	1	102	2
$\frac{1}{2}$	2,600	19	62	42	9,600	69	172	56
$\frac{1}{3}$	0	0	14	0	11,700	84	382	31

2. *The activity of the precipitates produced from one toxoid, (a) by varying the hydrogen ion concentration with acetic acid, (b) by saturating with ammonium sulphate.*

Experiments were next carried out to compare the efficiency of acetic acid and ammonium sulphate as precipitants. A typical experiment may be described. A volume of diphtheria toxoid was prepared and the acetic acid precipitation zone was determined, as described in a previous paper [Watson and Langstaff, 1926]. The p_H limits of this zone were found to be 3·1 to 4·35. Four litres of the toxoid were then precipitated at each of eight points within this zone and the precipitates recovered quantitatively by means of a Sharples' centrifuge. The total solids, total nitrogen content and the specific activity of the precipitate dissolved in alkali to p_H 8·0 were then estimated. A similar volume of the same toxoid (p_H 7·9) was completely saturated with ammonium sulphate and the activity of the precipitate dissolved in water estimated in the same way. The results are given in Fig. 1.

By varying the hydrogen ion concentration of the toxoid, maximum precipitation was produced at p_H 3·9, the isoelectric point of the active "protein" (Curve A). Correlated with this, the precipitate at this point was most active (Curve B). Curve C shows the concentration of Lf units per 20 mg. nitrogen of the precipitates produced at various points in the p_H zone 3·1–4·35. The maximum activity was 4100 units per 20 mg. nitrogen, and since the original toxoid contained 37 units per 20 mg. nitrogen a relative purification of 110 was effected by precipitation at the isoelectric point. Even at this point, however, only 39 % of the total active principle was recovered.

On the other hand, by completely saturating the toxoid (p_H 7·9) with ammonium sulphate, the whole of the activity was recovered in the precipitate (Curve D). As Seibert and Long [1926] have found with tuberculin, ammonium

sulphate is a far more energetic precipitant than acetic acid even at the isoelectric point. In fact a very strong resemblance (apart from heat stability) between the behaviour of the active principle of tuberculin and a true bacterial toxin such as diphtheria toxin may eventually be proved to exist.

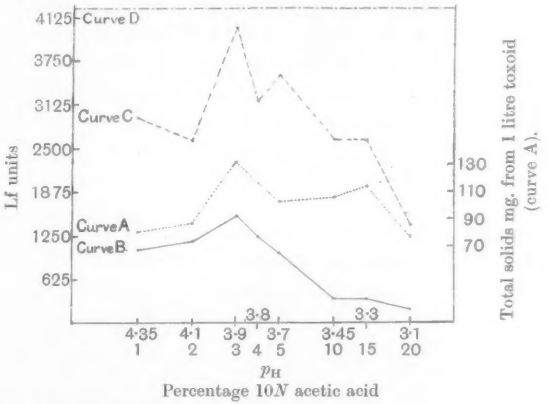

Fig. 1. The precipitability of a diphtheria toxoid by change of p_H with acetic acid and by ammonium sulphate.

Curve A. Total solids precipitated from 1 l. of toxoid at various c_H.

Curve B. Specific activity (Lf units) of various precipitates.

Curve C. Lf units per 20 mg. N of the precipitates.

Curve D. Specific activity (Lf units) of the precipitate produced by complete saturation of the toxoid (p_H 7·9) with ammonium sulphate.

3. *The influence of the preservative used for culture filtrates on the precipitability of the active principle.*

The particular chemical agent used for the destruction of the bacilli at the end of growth and the preservation of the culture filtrates during subsequent manipulation may exert a definite influence on the recovery of the active principle by precipitation with ammonium sulphate. The chief agents employed for this purpose are phenol, formaldehyde and toluene, but of these, toluene is the only one that apparently has no action on the active principle. Table II shows the recoveries from a series of culture filtrates treated with different preservatives.

In all cases except two (TB 182 and TX 179—beef-heart peptone media) the media were prepared by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927]. In the case of the filtrates treated with phenol, very little of the active principle is found in the precipitate, although examination of the amounts of nitrogen in the material precipitated in the case of unpreserved, toluene- or phenol-treated culture filtrates gave the same results. These low recoveries from phenol-treated filtrates may be due to the "salting out" of the antiseptic and the high concentration localised in the precipitate effecting severe destruction of the active principle [see Pope, 1927]. The

recoveries from unpreserved or toluene-treated filtrates are uniformly high and in some cases practically complete. On the other hand, the formalin-treated filtrates fall into two groups, one giving practically complete recovery and the other comparatively small. In every case where the yields were high the parent toxoid was well matured—*i.e.* the formalin had been in contact with the culture filtrates for a prolonged period and the amount of free formalin had been reduced to a minimum. Further, the p_H of the toxoid had been readjusted to 8·0. The low recoveries were produced from culture filtrates which had been in contact with formalin for a short time only. It would appear that the contributing cause to the low recoveries in these cases is a p_H effect. This effect is examined in the next section.

Table II. *The effect of the presence of preservative on the activity of the precipitate produced by complete saturation with ammonium sulphate.*

No.	Preservative	No. of Lf units per 100 cc.	Lf units recovered by complete saturation with $(NH_4)_2SO_4$	Percentage yield
TME 172	Formalin	1100	1050	96
TME 93	,,	425	425	100
CES 171 A	,,	1150	550	48*
TME 150	,,	1050	1000	95
TTE 154	Toluene	850	750	88
CES 169 A 3	,,	1900	1800	95
TTE 181	,,	1400	1200	86
TE 178 A	Phenol	1400	350	25
TE 177	,,	1600	500	31
TB 182	,,	1100	200	18
TX 179	Unpreserved	1200	1200	100
,,	Toluene	1200	1200	100
,,	Formalin	1200	600	50*
,,	Phenol	1200	200	17

* Formalin added a few hours before saturation with ammonium sulphate.

4. *The influence of the hydrogen ion concentration of the culture filtrates on the "salting out" effects of ammonium sulphate.*

Very few data exist in the literature on the salting out of proteins (except perhaps those of serum) by ammonium sulphate. It is probable that the effects produced with any one protein are characteristic only for that protein. With the object of testing the effect of salting out the *diphtheriae* "proteins" in acid and alkaline solutions, a series of culture filtrates were adjusted to different p_H and then saturated with ammonium sulphate. The activity of the precipitates was tested in the usual way. The results are collected in Fig. 2.

It will be seen that complete recovery of the active principle is usually found if the salting out is conducted in slightly alkaline solution (p_H 8·0). At points on the other side of neutrality the recovery may not be complete. In the acid precipitation zone (p_H *ca.* 4·0) very low recoveries are always experienced. This is probably caused by the partial acid precipitation of the

active principle in a form insoluble in water[1]. The whole mechanism of the salting out of proteins is an obscure one but it is of importance that the recovery by precipitation with ammonium sulphate of the active principle of diphtheria toxin in a form soluble in water is only complete in the absence of certain chemical substances (*e.g.* phenol or acid) which in some way affect the structure of the active principle.

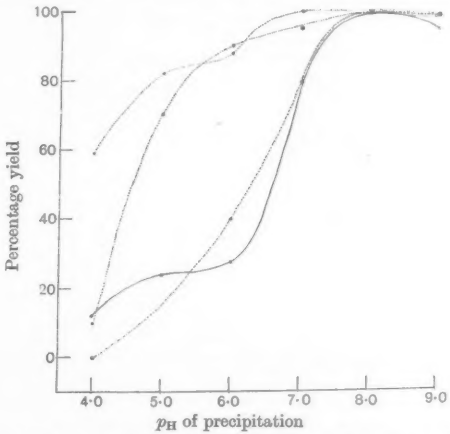

Fig. 2. The influence of p_H on the precipitation of culture filtrates by ammonium sulphate.

• – – • – – • Culture filtrate untreated.
——— Culture filtrate treated with 0·5 % toluene.
..... Culture filtrate treated with 0·75 % formalin.

5. *The chemical "purity" of the precipitates produced by acetic acid and ammonium sulphate.*

In any work involving the isolation and examination of substances of physiological interest it is obviously an advantage to utilise, as a basis for attacking the problem, material as free as possible from extraneous components. To obtain some idea of the relative purity of the precipitates during the course of the investigation, the concentrations of Lf units per mg. N of the original culture filtrates and the various precipitates have been determined. Different culture filtrates vary widely in their behaviour towards the same precipitant, but the general impression received during the course of the work was that although ammonium sulphate under certain conditions may completely precipitate the active principle from the culture filtrates, the precipitates produced in the acid precipitation zone contain relatively more Lf units per mg. N. The complete examination of a low value culture filtrate is shown in Table III. Acetic acid at the isoelectric point (p_H 3·9) produced a precipitate

[1] Mastin and Schryver [1926] have recently described a well-defined acidic denatured product of albumin soluble in dilute alkalis but insoluble in dilute acids.

which was 110·8 times as "pure" as the original toxoid. The "purest" precipitate obtained in the ammonium sulphate series was that produced by two-thirds saturation where a relative purity of 28·8 was obtained (compare Table I, where the precipitate produced at this level also contained the highest concentration of Lf units per mg. N).

Table III. *The relative purity of the acid- and the ammonium sulphate-precipitable material.*

Toxoid TME 93. 1·85 Lf units per mg. N

Precipitate in zone p_H 3·1–4·35			Ammonium sulphate precipitates		
p_H of pptn.	Lf units per mg. N pptd.	Relative purification of act. principle	Degree of saturation	Lf units per mg. N pptd.	Relative purification of act. principle
4·35	150	81·1			
4·1	132	71·4	Complete	17·7	9·6
3·9	205	110·8	$\frac{3}{4}$	47·2	25·5
3·8	163	88	$\frac{2}{3}$	53·2	28·8
3·7	180	97·3	$\frac{1}{2}$	27·2	14·7
3·45	132	71·3	$\frac{1}{3}$	No ppt.	–
3·3	132	71·3			
3·1	74	40			

6. *The chemical properties of the active substances.*

Apart from the presence of small traces of ammonium sulphate and of a larger amount of extraneous material, the chemical properties of the active substances obtained by salting out the culture filtrates are essentially similar to those of the acid-precipitable material previously described [Watson and Langstaff, 1926]. The salted out "proteins" can be freed from ammonium sulphate by dialysis, the loss of active principle depending largely on the nature of the membrane employed. The active material gives typical protein colour reactions and the specific substance is comparatively stable.

Summary.

1. The substance which causes the specific *in vitro* flocculation with antitoxin can be salted out of culture filtrates of *C. diphtheriae* by complete saturation with ammonium sulphate. Recovery is complete in the case of filtrates untreated with any chemical agent, and adjusted to p_H 8·0 before precipitation.

2. The presence of certain chemical agents (*e.g.* phenol or acid) prevents the complete recovery of the active principle by precipitation with ammonium sulphate.

3. The material salted out by ammonium sulphate is less chemically "pure" relative to the original culture filtrate than the acid precipitate produced at the isoelectric point although, in general, the chemical properties of the precipitates are the same.

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LXIII. UNSCALED FIBRES. A NEW ASPECT OF FIBRE RESEARCH.

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(*Received March 9th, 1927.*)

Hitherto both chemical and physical investigations on wool have dealt with the properties of the fibre as a whole. Conclusions drawn only from such observations as to how the structurally different parts (scale and cortex, and also medulla in the case of kempy fibres) are concerned in the total effect, can only be inferential. To resolve any property of the fibre into the contributory effects of scale and cortex requires removal of the scales in such a way as to leave the residual fibre core intact for separate investigation.

Chemical methods of separating scales and cortex almost certainly involve modifications in their chemical composition, and the examination of fibres so denuded of scales is of little value, so far as the specific effect of the cortex in the complete fibre is concerned. Mechanical separation is the only admissible method, and this can be successfully carried out in the manner described below, and as shown by the accompanying photomicrographs of fibres in various stages of removal of the scales and outer layers.

Method of unscaling.

Various methods were tried without success. Eventually it was found that by drawing the fibre with a small weight attached to keep it taut up and down across the edge of an ordinary microscope slide mounted horizontally, complete unscaling could be effected without breaking the fibre or damaging the bared interior. The slides require to be selected carefully. The narrow face must be smooth, and the two bounding edges require to be sharp and free from chipped places, which are easily detected when the finger nail is drawn along them. The severity of the scraping depends on the rate of rise and fall of the fibre, the departure from the vertical between the upper glass edge and the guiding eye placed above it, and the pressure on the glass edge as determined by the weight attached to the fibre. The scraping becomes more pronounced after the edge has been slightly roughened by friction with the fibre. The sound of the scraping is a good guide as to whether the operation is proceeding properly, or the edge is too much roughened for use. By slowly rotating the clip holding the upper end of the fibre while it is being raised and lowered, the weight also revolves and the whole surface becomes equally

scraped. Long coarse fibres are naturally best suited to the treatment, but with due precautions merino fibres can be unscaled with equal success. The scraped fibres have a different lustre, and no longer possess the property of "creeping" when rubbed lengthwise between the thumb and finger. This latter test affords a good indication of the progress of scale removal.

This simple apparatus meets the case where fibres require to be examined singly, for example, in swelling and elasticity investigations, and microscopic studies under various treatments, but for chemical analysis and other work requiring fibres in bulk, more rapid treatment is desirable. This can be effected by making the fibre pass a number of the glass edges suitably spaced, and by building a number of scraping units to treat several fibres simultaneously. An instrument on these lines is being developed, and will be described in a separate paper.

Appearance of unscaled fibres.

For the first series of photomicrographs (1, 2 and 3, Plate II), La Concordia Pure Lincoln fibres were used, these being of suitable length and uniformity, and showing a very clear scale structure in their natural condition. Euparal was used as mounting medium in preference to Canada balsam, as the former brings out the scale structure much more clearly. To give contrast the fibres were dyed (with Ponceau R.G.) before scraping.

The disappearance of scale edges and scale markings can be followed by microscopic observation. In the photographs shown, taken from a single fibre which had been subjected to thorough scraping, the middle portion of the fibre (3) is seen to have been considerably reduced in area without suffering, but rather gaining, in regularity of contour. The second photograph (2) shows a lesser degree of scraping, the unscraped end portion being shown on the extreme left (1).

The pores recently observed by Mark [1925] appear more distinctly and generally in increased numbers in the scraped portions. Probably the scales are more opaque than the cortex, and being also irregular in surface, scatter the light and obscure all but those pores immediately beneath them.

The effect of similarly treating Australian Merino fibres is shown in (4) and (5). The fibre cortex, while clearly defined and very regular, does not exhibit pores in the same striking manner as the Lincoln fibre.

The dyed fibres show scarcely any evidence of fibrillar structure in the cortex. Staining with Nile blue results in a striated appearance under the microscope suggesting narrow filaments closely packed, but not revealing any discrete cell structure. It is not impossible that these striae may be merely scratches made during the scraping, though this is regarded as improbable. The scraping is remarkably smooth under properly adjusted conditions, and fibres are rarely damaged. Where damage does occur, it is usually by lateral cuts due to horizontal vibration of the fibre along the glass edge, as shown in (6), Plate II.

By passing the fibre over a single edge and preventing any rotation, a plane surface can be produced on the fibre. By giving a half turn to the fibre clips the opposite side can be scraped, producing a thin ribbon, and interesting longitudinal sections are obtainable in this way. Number (7), Plate II, is a photomicrograph of a plane surface so produced with a La Concordia Lincoln fibre. The better definition thus obtained shows the pore structure much more clearly than is possible with a cylindrical surface.

Applications.

The production of such fibres opens up a wide field of investigation into the microscopic structure, chemical composition, behaviour towards reagents, and various physical properties of the fibre cortex as distinct from the complete fibre.

The foregoing observations are of a preliminary nature to indicate broadly the directions in which this method of attack may be of service in fibre research, on which lines investigations are now in hand.

Summary.

A method has been devised for the mechanical removal of the scales or outer layers of wool fibres in such a way as to leave the cortex or inner core intact for physical and chemical investigations. It consists essentially in drawing the fibre across the edge of a glass plate of suitable character, or over a series of such plates suitably arranged. The effectiveness of the method is illustrated by photomicrographs of wool fibres in various stages of scale removal. The method provides a means of ascertaining the chemical and physical characters of the cortex, as distinct from those of the fibre as a whole.

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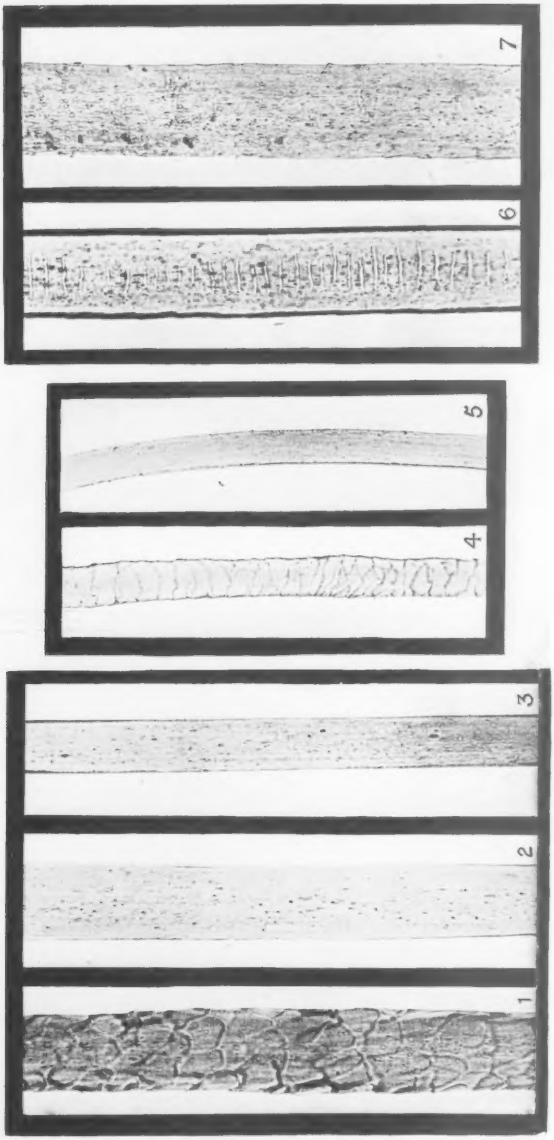

Magnification ×300.

UNIV OF MICH.

LXIV. NOTE ON THE EFFECT OF LIGHT UPON UROPORPHYRIN.

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(*Received February 1st, 1927.*)

Introduction.

The following paper gives some account of the phenomena occurring in relation to the action of light upon uroporphyrin, prepared from the urine of a case of haematoporphyria congenita.

It has been known for some time that uroporphyrin fades upon exposure to daylight, and Schumm [1916] has shown that, as the fading proceeds, the colour of the solution turns from rose-red to brown, and that an additional band appears in the absorption spectrum at 4610–4620 Å.

Apart from this, little seems to be known regarding the photochemistry of uroporphyrin, although a knowledge of it must be of importance in the investigation of the part played by uroporphyrin in the sensitisation to light that is so characteristic of haematoporphyria congenita.

Experimental.

In the course of some experiments dealing with the factors involved in the process of blistering as observed in the cases of haematoporphyria congenita, uroporphyrin solutions of different hydrion concentrations were exposed to daylight, and not only did a general fading take place, but there was also some differentiation of fading between the individual solutions.

With the purpose of gaining information concerning the relation between the degree of fading and the hydrion concentration of the solution, two series of experiments were performed, the one with crude uroporphyrin, the other with purified uroporphyrin.

(*a*) *Daylight effects.*

Series I. Crude uroporphyrin was prepared by Nebelthau's method [1899] from the urine of the case of haematoporphyria congenita described by Garrod and Mackey [1921]. This method consists essentially in the precipitation of uroporphyrin and other substances from the urine by acetic acid.

Solutions of the uroporphyrin in phosphate-buffered solutions were made up extending over the range p_H 6·2–8·2. The uroporphyrin was dissolved in $M/15$ Na_2HPO_4 solution in a concentration of 260 mg. per litre. To this was added, as shown in Table I, the necessary amounts of $M/1{\cdot}5$ KH_2PO_4 solution. In this way undue dilution of the uroporphyrin solution was avoided. (This

latter point was of importance, since colorimetric estimations had to be made, and economy had to be exercised as regards the amount of uroporphyrin used.

The p_H of each solution was confirmed by electrometric determinations.

Table I.

Solution No. ...	1 cc.	2 cc.	3 cc.	4 cc.	5 cc.
$M/15$ Na_2HPO_4 + Up. ...	4	4	4	4	4
$M/1{\cdot}5$ KH_2PO_4	1·6	·4	·1	·02	—
Water	·4	1·6	1·9	1·98	2
p_H (±0·1)	6·2	6·8	7·3	7·7	8·2

The solutions were in every case made up in a dark room where the stock solution of uroporphyrin in Na_2HPO_4 was kept. Before exposure to light the solutions were of a bright rose-red colour.

For experiment, the solutions were exposed in sets of five in Pyrex glass tubes to daylight of varying intensity and for different times. (An ordinary photographic exposure meter was used in some cases for comparing the intensities of the different lights, but its use was afterwards discontinued, since the light rarely remained of constant intensity for more than a few minutes at a time.)

After exposure the degree of fading of the solutions was estimated colorimetrically.

Upon exposure to light a general fading always took place throughout the series, the rose colour tending to turn brownish and lighter. This fading was not due to precipitation of the uroporphyrin. Some difficulty was experienced in obtaining trustworthy colorimetric readings after exposure, as the "browning" referred to above made the colour matches with the control solutions very bad and hard to get with any great degree of accuracy. In every case, however, it was found that the solutions at p_H 6·8–7·3 differed most in colour from the control tube.

Series II. In order to make certain upon this point, a further amount of uroporphyrin was prepared from the same source by the method of Fischer and Zerwecke [1924]. By this method uroporphyrin is obtained free from traces of coproporphyrin, and much less "browning" is observed during and after exposure, the final colour being much purer. The above experiments were then repeated exactly in detail with the purified uroporphyrin, when much more constant results were obtained, and the colour matches were far better. Table II illustrates a typical experiment.

Results obtained from an experiment using purified uroporphyrin exposed for 1 day to north light in Pyrex tubes:

Table II.

Solution No.	1 cc.	2 cc.	3 cc.	4 cc.	5 cc.	Control cc.
Absolute colorimetric reading	18·1	18·4	18·9	18·4	18·2	15
Reading in % increase over control	20·6	22·6	26·0	22·6	21·3	—
p_H (±0·1)	6·2	6·8	7·3	7·7	8·2	—

(*b*) *Effects of ultraviolet light.*

Upon exposure of solutions, made up as above, to ultraviolet light (Cooper-Hewitt lamp) in quartz tubes, a general fading, irrespective of p_H, was obtained.

No evidence of differentiation was found with either the crude or the purified uroporphyrin.

(*c*) *Effects of exposure to light in absence of oxygen.*

In order to determine whether these effects might be due to oxidation, sets of solutions were sealed off in nitrogen, the technique used being similar to that described by Ellis and Newton [1925]. They were then exposed to light. After exposure gas samples from each tube were taken, and the oxygen content determined. The percentage of oxygen found was never greater than 0·1.

Under these circumstances a difference was noted between the behaviour of crude and purified uroporphyrin.

Pure uroporphyrin solutions faded with occurrence of the usual differentiation, and no difference was detected between their behaviour and that of controls set up in tubes open to the atmosphere. It would therefore appear that oxidation plays little or no part in the phenomena of fading described. Crude uroporphyrin solutions, on the other hand, fade slowly in absence of oxygen, but quickly if oxygen be present, at a much greater rate than do solutions of pure uroporphyrin. It is evident that oxidation plays some part in the fading, and it is possible that there are two reactions going on, one a purely photochemical reaction, which occurs with both purified and crude uroporphyrin, and does not involve oxidation, and the other an oxidative reaction, occurring only with the crude uroporphyrin solution.

(*d*) *Spectral effects.*

As the solutions fade there is little or no change in the position of the absorption-bands (of which there are four—in the solution of the purified uroporphyrin at 496–513, 533–547, 557–573, 607–618 $\mu\mu$, and in the crude uroporphyrin at 498–525, 537–558, 565–575, 607–618 $\mu\mu$), at least as far as could be determined with a small direct-vision spectrometer.

The bands simply fade away as the solution fades. Within the range of p_H used the appearance of the "Kalilichtreaktion" band described by Schumm at 461 $\mu\mu$ could not be detected.

Conclusion.

It will be seen from the above results that the degree of fading does not increase or decrease progressively with the change in reaction of the solution. It has constantly been found that there is some definite difference in the degree of fading at p_H 7·2–7·6. Though this is not great as compared with the general fading, it has been observed too constantly to be attributable to experimental error.

Whether it has any significance cannot as yet be said, but it is interesting that it should occur at a reaction which approximates to the physiological reaction. It may have some bearing upon the fact that the teeth of the patient, from whose urine the uroporphyrin was obtained, were in March 1918 (when he was two years old) of a pink colour hardly to be distinguished from that of the gums, but three years later had undergone a colour change to brownish-pink.

So far it has not been possible to obtain results in confirmation of the above by using solutions with other buffers.

SUMMARY.

The fading of solutions of uroporphyrin prepared from a case of haematoporphyria congenita has been investigated within the p_H range 6·2–8·2.

1. Over this range there is a differential fading, which reaches a maximum at p_H 7·3 approximately.

2. The rate of the general fading of uroporphyrin solutions is greater in the case of those containing crude uroporphyrin than in those containing purified uroporphyrin.

3. In the case of crude uroporphyrin, the fading is in part associated with the presence of oxygen; for the fading of pure uroporphyrin the presence of oxygen is not necessary.

I have to thank Prof. Sir Archibald E. Garrod and Dr L. Mackey for an introduction to the case of haematoporphyria congenita; I am also indebted to Prof. R. A. Peters for much kindly criticism and advice.

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LXV. INFLUENCE OF GLUCOSE, ALCOHOL AND CARBON DIOXIDE AT BAROMETRIC PRESSURE ON THE p_H VALUES OF PHOSPHATE AND BICARBONATE SOLUTIONS, DETERMINED BY MEANS OF HYDROQUINHYDRONE ELECTRODES.

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I. Introduction.

Liquids in the state of fermentation are generally saturated with CO_2. The determination of the p_H of such liquids, therefore, presents certain difficulties, though the liquids have a rather heavy buffer effect and well defined p_H values.

As a matter of fact, hydrogen electrodes cannot be used for the determination of p_H in liquids saturated with CO_2 at a pressure of one atmosphere, and if colorimetric methods are used, special care must be taken to keep the liquids saturated with CO_2 during the measurements.

On the other hand, it seemed possible that the quinhydrone electrode [Biilmann, 1921, 1923] would be suitable for the determination of p_H in liquids saturated with CO_2, as this electrode acts as a hydrogen electrode with a very feeble hydrogen pressure. For our purpose it will be sufficient to remember that the quinhydrone acts as a hydrogen electrode, in which hydrogen and platinum black are substituted by quinhydrone and bright platinum. The quinhydrone dissolves in water and dissociates into quinol, $C_6H_4O_2H_2$, and quinone, $C_6H_4O_2$. Quinol acts as hydrogen by the reaction

$$C_6H_4O_2H_2 - 2\theta \rightarrow C_6H_4O_2 + 2H^+,$$

which substitutes the reaction

$$H_2 - 2\theta \rightarrow 2H^+$$

in the hydrogen electrode.

The inverse reaction

$$2H^+ + C_6H_4O_2 + 2\theta \rightarrow C_6H_4O_2H_2$$

also takes place. It corresponds to the reaction

$$2H^+ + 2\theta \rightarrow H_2,$$

produced in the hydrogen electrode by the catalytic action of the platinum black.

In dilute solutions the potential of the quinhydrone electrode is a linear function of the p_H value, similar to that of the potential of the hydrogen electrode. At higher concentrations the influence of the solutes on the activities of quinol and quinone, formed in equimolar concentrations by the dissociation of quinhydrone, may have a certain influence on the potential, because the reversible reaction in the cell is a transformation of *dissolved* quinol into *dissolved* quinone and *vice versa*, so that the potential does not in this case depend exclusively on p_H. This influence also interferes with our experiments, which deal with solutions containing up to 10 % glucose, but it is eliminated in the quino-quinhydrone electrode and the hydroquinhydrone electrode, described by Biilmann and Lund [1923]. For our purpose, we have only to mention that in the hydroquinhydrone electrode the liquid under examination is *saturated* both with quinol and with quinhydrone, and consequently, as shown in the quoted papers, the electrode reaction is here a transformation of *solid* quinol into *solid* quinhydrone. As the liquid is kept saturated with both these substances, their activities are constant, and this is why the relation between the potential and p_H is linear and independent of the concentration of other substances dissolved in the liquid.

Our experiments revealed a specific influence of glucose on the platinum plates of the electrodes, when the simple quinhydrone electrode was used. This interesting phenomenon is treated in Section VII. For the study of the influence of glucose and alcohol on the p_H values of buffer solutions and of the determination of p_H in solutions saturated with CO_2, we used the hydroquinhydrone electrode.

First of all we established, by means of phosphate buffer solutions, that the electrodes are reproduced with well-defined and stable potentials. By these experiments we arrived at a comparison electrode of the same type and almost the same p_H as the other electrodes examined, which gives our determinations the advantage that the measured cells have either no liquid junction potentials at all or only very feeble liquid junction potentials, and that the determinations do not depend on determinations of potentials between the comparison (standard) electrodes and other electrodes, but directly on the thermodynamical equation

$$p_H = p_{H_1} - \frac{\pi}{0{\cdot}0001983 \times T},$$

in which p_H is the p_H value of the examined liquid, p_{H_1} that of the standard electrode, and π indicates how much the potential of the examined electrode is higher than that of the standard electrode. Consequently, the higher the observed potential, the more acid the examined liquid, and the lower the p_H value.

II. Standard hydroquinhydrone electrode.

Test of method.

The electrolyte of the standard electrode is a mixture of equal volumes of 1/15 *M* Na_2HPO_4 and 1/15 *M* KH_2PO_4. By direct measurement its p_H value was found to be 6·79.

For the electrodes were used vessels as described by Biilmann and Lund [1923] and shown in Fig. 1. 10 cc. phosphate solution shaken with a pulverised mixture of 1 g. quinol and 0·1 g. quinhydrone were put in the vessel, and the platinum spiral was placed in the narrow part of the vessel. The electrode was left in the thermostat at 18° and measured against another electrode of the same kind, next day against a newly prepared electrode and so on. The two electrodes were joined by means of a 3·5 *M* KCl bridge. The results are shown in Table I.

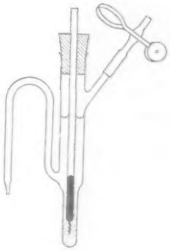

Fig. 1.

Table I.

Days	$\Delta\pi$	Δp_H
0	−0·00002	+0·003
1	+0·00040	−0·007
2	−0·00024	+0·004
4	−0·00005	+0·001

It is seen from the table that the changes in potential correspond to changes Δp_H which during the first 4 days are quite negligible.

Further we examined the reproducibility and steadiness of similar electrodes containing 10 g. glucose in 100 cc. Table II shows the potentials of two such electrodes measured against the phosphate standard electrode at 18°.

Table II.

(+) 1/15 *M* phosphate, p_H 6·79 + glucose, 10 g. in 100 cc. | 3·5 *M* KCl | 1/15 *M* phosphate, p_H 6·79 (−)

	Volt	
Minutes	I	II
0	+0·0077	+0·0079
10	+0·0074	+0·0078
30	—	+0·0078
35	+0·0074	—
65	—	+0·0078
70	+0·0075	—
1020	—	+0·0075
1080	+0·0073	—

The figures show that the maximum difference between the potentials of the two glucose-phosphate electrodes, namely between 0·0079 for electrode II at time 0 and 0·0073 for electrode I at time 1080, corresponds to less than 0·01 in the p_H value.

The reproducibility and stability of the hydroquinhydrone electrodes in a glucose-phosphate solution therefore proved to be very good.

III. Influence of glucose on p_H of phosphate mixture.

The figures in Table II show that glucose has an influence on the p_H value of the phosphate mixture. We therefore examined the influence of different concentrations of glucose on the phosphate mixture. The mixtures were prepared from stock solutions of primary and secondary phosphate in double concentrations (2/15 *M*), carbon dioxide-free water being used. Table III shows the results obtained with mixtures in which the concentration of glucose varied from 10 g. in 100 cc. to 1/32 of this value. The measurements were carried out at 18° and 25°, and the potentials recorded in the table are corrected for liquid junction potentials by means of 3·5 *M* and 1·75 *M* KCl. The corrections were between 0·0005 and 0·0000 volt. (It is because of this correction that the value for 10 g. glucose in Table III is higher than the corresponding value in Table II, where it is given without correction.)

Table III.

(+) 1/15 *M* phosphate, p_H 6·79 + glucose | KCl | 1/15 *M* phosphate, p_H 6·79 (−)

	18°		25°	
g. glucose in 100 cc.	π	Δp_H	π	Δp_H
10	+0·0081	−0·140	+0·0081	−0·137
5	+0·0042	−0·073	+0·0043	−0·071
2·5	+0·0024	−0·042	+0·0023	−0·039
1·25	+0·0017	−0·029	+0·0017	−0·029
0·625	+0·0012	−0·021	+0·0012	−0·020
0·3125	+0·0008	−0·014	+0·0009	−0·015

We first observe that the electrodes *with* glucose are positive to the electrodes *without* glucose, which means that *glucose makes the mixture more acid*. The influence Δp_H is not negligible. The acidity of glucose itself is so feeble that glucose does not act as an acid at p_H 6·79. The influence on the acidity may be explained as an influence on the activity of the water of the solutions.

Further we see that the influence on the potential is independent of the temperature. Concerning this phenomenon, it is to be remembered that as the hydration of the hydrions is expressed by the equation

$$H^+ + mH_2O \rightleftarrows H^+, mH_2O,$$

we have

$$\frac{a'_{H^+}}{a^\circ_{H^+}} = \left(\frac{a^\circ_{H_2O}}{a'_{H_2O}}\right)^m = \left(\frac{P^\circ}{P'}\right)^m,$$

where a'_{H^+}, $a^\circ_{H^+}$, a'_{H_2O} and $a^\circ_{H_2O}$ represent the activities of hydrions and water in two solutions, P° and P' the water vapour pressures of these solutions.

According to the van 't Hoff-Raoult law P°/P' is independent of the temperature. The curve I in Fig. 2 shows a linear relation between π and g. glucose over a large range of concentrations. This is due to the fact that for the concentrations of glucose examined the values of log P°/P' calculated by means of the van 't Hoff-Raoult law, are proportional to the concentrations:

g. glucose in 100 cc.	10	5	2·5	1·25	0·625	0·3125
log $P^\circ/P' \times 10^5$	432	217	108	54	26	13

Finally we have compared the electrometric measurements with colorimetric determinations by means of bromothymol blue. We found:

g. glucose in 100 cc.	10	5	2·5	1·25	0
p_H electrometric	6·65	6·72	6·75	6·76	6·79
p_H colorimetric	6·71	6·75	6·78	6·79	6·79

The curve IV (Fig. 2) represents measurements made by Schreiner [1924] with saccharose in 0·005 *M* HCl and shows the influence of saccharose on the acidity of the solution, measured by the potential of a hydroquinhydrone electrode. The curve is drawn so that the abscissae correspond to equimolar concentrations of saccharose and glucose. This curve is almost parallel with our glucose curve.

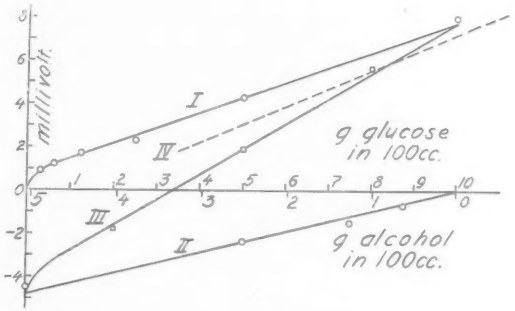

Fig. 2.

IV. Influence of alcohol on the p_H of phosphate mixture.

Experiments with mixtures of phosphate solution and alcohol were made in the same way as with glucose. Regarding the amounts of alcohol formed by fermentation of glucose, alcohol was applied in concentration varying from about 5 g. in 100 cc. to 1/8 of this amount. The results are recorded in Table IV.

Table IV.

(−) 1/15 *M* phosphate, p_H 6·79 + alcohol | KCl | 1/15 *M* phosphate, p_H 6·79 (+)

	18°		25°	
g. alcohol in 100 cc.	π	Δp_H	π	Δp_H
5·1	−0·0044	0·076	−0·0045	0·076
2·55	−0·0020	0·035	−0·0024	0·041
1·275	−0·0015	0·026	−0·0015	0·025
0·6375	−0·0006	0·010	−0·0007	0·012

The values in Table IV are corrected for liquid junction potentials. The highest extrapolation was − 0·0003 volt. First we observe that the influence of alcohol is the opposite of the influence of glucose. The electrode containing alcohol is here the negative one, which means that it is less acid than the same

electrode without alcohol. So we have not to deal with an ordinary influence of a solute on the activity of the water, and the whole matter may be considered rather complicated. On the other hand, we observe here as with glucose an influence practically independent of the temperature. The influence Δp_H on p_H is of the same order as in the case of glucose, but has the inverse sign. In Fig. 2, curve II shows the relation between g. alcohol and π.

V. Phosphate mixtures containing both glucose and alcohol.

Finally we have examined mixtures containing both glucose and alcohol. The mixtures are composed according to the transformation of a glucose solution into alcohol by fermentation. The measurements are shown in Table V.

Table V.

1/15 *M* phosphate, p_H 6·79 + glucose and alcohol | KCl | 1/15 *M* phosphate, p_H 6·79

Glucose g. in 100 cc.	Alcohol g. in 100 cc.	18°	25°	25° calculated
8·0	1·02	+0·0056	+0·0057	+0·0055
5·0	2·55	+0·0020	+0·0019	+0·0020
2·0	4·08	−0·0020	−0·0018	−0·0016

The influence of glucose and alcohol is almost additive. The calculated values of π agree with the observed within 0·2 millivolt. For the calculation are used values obtained by graphic interpolation on curve III in Fig. 2. It represents the sum of the curve for glucose from 0 to 10 g. in 100 cc. and the curve for alcohol from 5·1 to 0 g. in 100 cc. The squares round this calculated curve show the places of the observed values of π from Table V.

VI. Liquids saturated with carbon dioxide.

The following experiments deal with solutions of disodium hydrogen phosphate and of sodium bicarbonate saturated with carbon dioxide. The arrangement is shown in Fig. 3. First of all the carbon dioxide passed through a wash-bottle containing a solution of sodium bicarbonate, then a wash-bottle *a* containing some of the examined liquid, whereupon it was led into the electrode

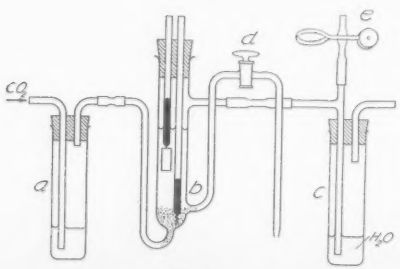

Fig. 3.

vessel *b*, which was constructed as a simple hydrogen-electrode vessel. *a* and *b* were placed in the same thermostat, so that the carbon dioxide was saturated with water vapour at such a pressure that no condensation or evaporation of water was caused by the passage of the gas through the electrode vessel. From *b* the carbon dioxide passes through the vessel *c*, which contains a little water and acts as an air-trap. If higher pressures than the actual barometric pressure were required, the carbon dioxide was led to the bottom of a high glass cylinder filled with water or salt solution.

The electrode vessel contains two electrodes: in the upper part of the liquid a platinum plate, in the lower part a platinum spiral. The latter is placed in the paste of the liquid and solid quinol and quinhydrone. For 25 cc. solution were used 3 g. of a mixture of 10 g. quinol and 1 g. quinhydrone. The liquids were saturated with CO_2 before addition of the quinol-quinhydrone mixture, and then the saturation with CO_2 was continued until the potentials of the platinum plate and the platinum spiral agreed within 0·0001 volt. Then the potential of the electrode was measured against the phosphate standard electrode (p_H 6·79). If the stop-cock *d* of the electrode vessel is closed during the measurement, the conductivity will be very feeble. Therefore the carbon dioxide supply is cut off, the clip at *e* is opened for a moment, the stop-cock *d* is opened and the reading is taken. When the electrode vessel was not shaken by these manipulations, the liquid showed a constant and reproducible potential, even if it were saturated with CO_2 at a pressure higher than the actual barometric pressure.

The CO_2-pressures indicated in the following tables are the pressures of *dry* CO_2. The potentials are corrected for liquid junction potentials in the ordinary way.

(*a*) *Influence of the CO_2-pressure on p_H.*

First we examined the influence of variations in the CO_2-pressure on the p_H-values of secondary phosphate solutions saturated with CO_2. The experiments were carried out with 0·1 *M* Na_2HPO_4, and the CO_2-pressure varied within the limits of the usual variations of the barometric pressure.

Table VI.

18°. 0·1 *M* Na_2HPO_4

CO_2, mm. Hg	π	p_H
733	+0·0200	6·444
746	+0·0204	6·437
755	+0·0214	6·419
774	+0·0224	6·402
792	+0·0230	6·392

The examined electrode is positive to the standard electrode.

In Fig. 4, the pressure of carbon dioxide is plotted against p_H. The relation is seen to be almost linear. None of the observed values of p_H differs more than 0·005 from the values given by the curve. For 0·025 *M* and 0·200 *M* Na_2HPO_4 the change in π was of the same order.

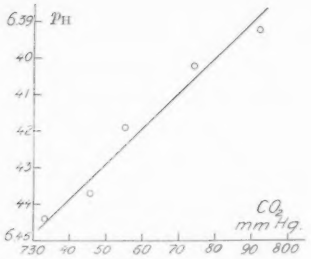

Fig. 4.

Fig. 5.

(*b*) *Reproducibility of the electrodes.*

The reproducibility of the electrodes saturated with CO_2 is shown in Table VII. Different solutions of Na_2HPO_4 and of $NaHCO_3$, containing 5 g. glucose in 100 cc., were measured against the standard electrode with p_H 6·79 at 25°.

Table VII.

Na_2HPO_4		mm. CO_2	π
0·2 *M*	...	733	+0·0112
,,		728	+0·0111
0·1 *M*	...	733	+0·0201
,,		728	+0·0192
$NaHCO_3$			
0·05 *M*	...	746	+0·0179
,,		743	+0·0178
0·025 *M*	...	743	+0·0335
,,		738	+0·0335

The examined electrode is positive to the standard electrode.

It did not seem necessary to compare the different electrodes at exactly the same pressure, as the differences of the pressures will only correspond to one or two tenths of a millivolt. The experiments then show that electrodes saturated with CO_2 are reproduced very exactly.

(*c*) *Influence of glucose on the p_H of phosphate solutions saturated with CO_2.*

In the same way we have examined a series of solutions of Na_2HPO_4, in concentrations varying from 0·025 to 0·5 *M*, without glucose or with 5 g. glucose in 100 cc. The measurements were made at 18° and at 25°. Further, at 25° the p_H values of the solutions were determined with bromothymol blue (BTB) and with bromocresol purple (BCP) in order to obtain a comparison between the electrometric and colorimetric determinations of p_H in liquids saturated with CO_2.

The colorimetric measurement was made in the following way. 10 cc. of the solution saturated with CO_2 were placed in a 20 cc. test-tube, to the bottom of which was attached a narrow tube as shown in Fig. 5. Five drops of the indicator solution were added, the test-tube was placed in the thermostat at the requisite temperature, and CO_2 was bubbled through the liquid in the

test-tube (as hydrogen in a hydrogen electrode). First of all the CO_2 was washed with $NaHCO_3$ solution and then with some of the liquid under examination, as in the electrometric experiments, in order to prevent evaporation of water from the tested solution. When the saturation with CO_2 was complete, the colorimetric determination was made in the ordinary way. For the comparison we used Sørensen's phosphate buffer mixtures; the exact p_H values of the mixtures were determined with the simple quinhydrone electrode.

The results are recorded in Tables VIII–X. In all the measurements the examined electrode was positive to the standard electrode.

Table VIII.

18°. Without glucose

					735 mm. CO_2	
Na_2HPO_4		mm. CO_2	π	p_H	π	p_H
0·025 *M*	...	737	+0·0431	6·04	+0·0430	6·05
0·050 *M*	...	737	+0·0306	6·26	+0·0305	6·26
0·100 *M*	...	749	+0·0204	6·44	+0·0196	6·45
0·200 *M*	...	749	+0·0122	6·58	+0·0114	6·59

The figures of the third and fourth columns give the directly measured potentials and the corresponding values of p_H. In order to compare the values, it is necessary to refer them to the same CO_2-pressure. This is done by means of the linear relation between the potential, or the p_H, and the pressure shown in Table VI and Fig. 4. It may be supposed that the small corrections are almost the same for the different concentrations of phosphate, at least within the accuracy of the measurements. The values of π and p_H referred to an average pressure of 735 mm. are recorded in the last two columns of the tables.

Table IX.

25°. Without glucose

					735 mm. CO_2	
Na_2HPO_4		mm. CO_2	π	p_H	π	p_H
0·025 *M*	...	728	+0·0399	6·11	+0·0403	6·11
0·050 *M*	...	728	+0·0272	6·33	+0·0276	6·32
0·100 *M*	...	722	+0·0175	6·49	+0·0182	6·48
0·200 *M*	...	722	+0·0097	6·62	+0·0104	6·61
0·500 *M*	...	735	+0·0025	6·75	+0·0025	6·75

Table X.

25°. With 5 g. glucose in 100 cc.

					735 mm. CO_2	
Na_2HPO_4		mm. CO_2	π	p_H	π	p_H
0·025 *M*	...	739	+0·0410	6·10	+0·0408	6·10
0·050 *M*	...	739	+0·0292	6·30	+0·0290	6·30
0·100 *M*	...	728	+0·0192	6·47	+0·0196	6·46
0·200 *M*	...	733	+0·0112	6·60	+0·0113	6·60
0·500 *M*	...	738	+0·0040	6·72	+0·0038	6·73

The measurements show that in the case of phosphate solutions saturated with CO_2, glucose has only a very feeble influence on the potential and the p_H.

The differences between the potentials of solutions without and with glucose do not seem to depend on the concentration of phosphate. The variations of p_H are nearly parallel in the three series of measurements:

Na_2HPO_4, M ...	...	0·025	0·050	0·100	0·200	0·500
18°	p_H	6·05	6·26	6·45	6·59	—
25°	„	6·11	6·32	6·48	6·61	6·75
25°, with glucose	„	6·10	6·30	6·46	6·60	6·73

In the solution of secondary phosphate saturated with CO_2, we have a mixture of secondary and primary phosphate and of bicarbonate, according to the equilibrium

$$HPO_4^{--} + CO_2 + H_2O \rightleftarrows H_2PO_4^- + HCO_3^-.$$

Experiments to be described later show that the phosphate solution saturated with CO_2 does not change its p_H value so much by dilution as a pure bicarbonate solution saturated with CO_2.

We have carried out a comparison between the electrometric and the colorimetric determination of p_H in solutions saturated with CO_2. The method has already been described in the introduction to this section of our paper. For the comparison, the electrometric determinations are referred to the CO_2-pressures at which the colorimetric determinations were made. The values are recorded in Table XI.

Table XI. *Comparison of electrometric and colorimetric determinations at* 25°.

	Without glucose				With 5 g. glucose in 100 cc.			
		p_H				p_H		
			Colorim.				Colorim.	
Na_2HPO_4 M	mm. CO_2	Electr.	BTB	BCP	mm. CO_2	Electr.	BTB	BCP
0·025	744	6·09	6·00	6·00	744	6·09	6·00	6·00
0·050	729	6·33	6·22	6·22	729	6·31	6·22	6·22
0·100	729	6·48	6·44	6·45	729	6·47	6·44	6·45
0·200	744	6·60	6·65	6·65	744	6·59	6·65	6·70
0·500	738	6·75	6·96	—	738	6·72	6·96	—

The measurements show that the colorimetric method gives lower p_H values than the electrometric one up to 6·4; above this the colorimetric method gives higher values than the electrometric one.

(*d*) *Influence of glucose on the p_H of bicarbonate solutions saturated with CO_2.*

The experiments were made exactly as the experiments with phosphate. The bicarbonate solutions were prepared from solutions of sodium hydroxide by saturation with CO_2. The measurements are recorded in Table XII. In the last two columns of these tables are shown the p_H values referred to a pressure of 740 mm. A comparison between the p_H values of the two series of solutions shows that the influence of glucose on the acidity is very feeble, and that the bicarbonate solutions with glucose seem *less acid* than the solutions without glucose.

Table XII.

25°. Without glucose

$NaHCO_3$		mm. CO_2	π	p_H	p_H at 740 mm.
0·025 *M*	...	738	+0·0335	6·22	6·22
0·050 *M*	...	725	+0·0190	6·47	6·46
0·100 *M*	...	756	+0·0036	6·73	6·74
0·200 *M*	...	756	−0·0109	6·97	6·98
0·500 *M*	...	755	−0·0284	7·27	7·28
			With 5 g. glucose in 100 cc.		
0·025 *M*	...	738	+0·0335	6·22	6·22
0·050 *M*	...	746	+0·0179	6·49	6·49
0·100 *M*	...	754	+0·0032	6·74	6·75
0·200 *M*	...	756	−0·0109	6·97	6·98
0·500 *M*	...	755	−0·0276	7·26	7·27

When we make a comparison of colorimetric and electrometric determinations of p_H (Table XIII), the last mentioned being referred to the pressures at which the colorimetric determinations are made, it shows almost the same cross point for the two methods as disodium hydrogen phosphate saturated with CO_2.

Table XIII.

	Without glucose					With glucose			
		p_H				p_H			
			Colorim.				Colorim.		
$NaHCO_3$ *M*	mm. CO_2	Electr.	BTB	BCP	mm. CO_2	Electr.	BTB	BCP
0·025	738	6·22	6·00	6·06	738	6·22	6·00	6·06
0·050	725	6·47	6·33	6·35	725	6·50	6·33	6·35
0·100	756	6·73	6·71	6·79	754	6·74	6·71	6·79
0·200	756	6·97	7·00	—	756	6·97	7·00	—
0·500	755	7·27	7·40	7·40*	755	7·26	7·40	7·40*

* Cresol red.

Finally we have tried to verify our determinations with bicarbonate in the following way.

The action of CO_2 on H_2O is

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+,$$

whence

$$a_{HCO_3^-} \times a_{H^+} = k \times a_{CO_2} \times a_{H_2O},$$

where a_{CO_2}, a_{H_2O}, $a_{HCO_3^-}$ and a_{H^+} represent the activities of CO_2, H_2O, HCO_3^- and H^+. If the pressure P_{CO_2} of CO_2 is constant, and as a_{H_2O} may be supposed to vary but very little in comparison with the other activities, we get

$$a_{HCO_3^-} \times a_{H^+} = k'.$$

For a_{H^+} we have

$$p_H = -\log a_{H^+} + 0{\cdot}04.$$

As for $a_{HCO_3^-}$, it may be calculated from the concentrations of $NaHCO_3$ by means of the activity coefficient for HCO_3^-. According to Warburg [1922] $\log f_a(HCO_3^-) = -0{\cdot}46\sqrt[3]{c}$, where c is the stoichiometric concentration of bicarbonate.

The bicarbonate solutions are measured at different CO_2-pressures. In Table XII p_H values referred to 740 mm. are also to be found. This reference is made by calculation. We have at two pressures P_{CO_2} and P'_{CO_2} (when $a_{HCO_3^-}$ is constant)

$$\frac{P_{CO_2}}{P'_{CO_2}} = \frac{a_{H^+}}{a'_{H^+}},$$

or

$$-\log a'_{H^+} = -\log a_{H^+} + \Delta,$$

where

$$\Delta = \log \frac{P_{CO_2}}{P'_{CO_2}}.$$

In Table XIV are shown the values of $\log (a_{HCO_3^-} \times a_{H^+})$, calculated from the experiments in Table XII.

Table XIV.

$NaHCO_3$	$a_{HCO_3^-}$	p_H	$-\log a_{H^+}$	$-\log (a_{HCO_3^-})$	$-\log (a_{HCO_3^-} \times a_{H^+})$
0·025 *M*	0·0179	6·22	6·26	1·747	$8·00_7$
0·050 *M*	0·0339	6·46	6·50	1·471	$7·97_1$
0·100 *M*	0·0612	6·74	6·78	1·214	$7·99_4$
0·200 *M*	0·1076	6·98	7·02	0·968	$7·98_8$
0·500 *M*	0·2157	7·28	7·32	0·667	$7·98_7$

We see that the constancy of $-\log (a_{HCO_3^-} \times a_{H^+})$ is very good. In the calculation we presume that the change of the activity of water may be considered constant. This may not be quite right. But the influence will be very feeble. If we suppose that the influence of the bicarbonate on the activity of the water is nearly the same as of an equimolar solution of potassium chloride, and if we introduce this influence in the calculation we arrive at the following values:

$NaHCO_3$	0·025	0·050	0·100	0·200	0·500 *M*
$-\log (a_{HCO_3^-} \times a_{H^+})$ ...	$8·00_7$	$7·97_1$	$7·99_5$	$7·99_1$	$7·99_4$

Further in a single case we have found agreement between the calculation of the influence of the CO_2-pressure on p_H and the influence observed experimentally. A 0·1 *M* solution of $NaHCO_3$ was measured with the hydroquinhydrone electrode in the ordinary way.

CO_2-pressure	p_H	Δp_H Found	Δp_H Calculated
742 mm.	6·669		
778 mm.	6·648	$0·02_1$	$0·02_{06}$

VII. Measurements with the simple quinhydrone electrode.

In the experiments described above we used the hydroquinhydrone electrode. The following were made with the simple quinhydrone electrode.

First we examined electrodes made with solutions of quinhydrone in 0·01 *N* HCl, 0·09 *N* KCl, with or without glucose. Accordingly we measured cells of the type

$$(+) \text{ acid} + \text{glucose} \mid \text{KCl} \mid \text{acid } (-),$$

and we always found the electrodes with glucose to be more positive than the electrodes in the same electrolyte without glucose.

As, in the case of the simple quinhydrone electrode, there might perhaps be an influence not only on the activity of the hydrion, but also on the activity of the components of the quinhydrone, we examined also the solution with the hydroquinhydrone electrode, which only shows the influence on the activity of the hydrions. The results are recorded in Table XV.

Table XV.

25°. 0·01 *N* HCl, 0·09 *N* KCl

g. glucose in 100 cc.	Quinhydrone π		Hydroquinhydrone π
10	0·0043	0·0043	0·0041
5	0·0023	0·0022	0·0018
2·5	0·0010	0·0012	0·0012
1·25	0·0004	0·0005	—

We see that the potentials agree well and that also in the simple quinhydrone electrode we have only an influence on the activity of the hydrion.

For 0·1 *N* HCl we found at 25°

10 g. glucose in 100 cc. 0·0042 volt
5 ,, 0·0021 ,,

values which agree completely with the figures in Table XV. The curve representing the relation between g. glucose and π is a straight line.

During our measurements we observed that hydroquinhydrone electrodes made with 0·01 *N* HCl, 0·09 *N* KCl are more stable in the presence of glucose than without glucose. While the hydroquinhydrone in this electrolyte was found to change 2 millivolts when kept from one day to the day after, it was quite stable in the presence of 5 % glucose. Two electrodes containing 5 g. glucose in 100 cc. of this electrolyte were kept at 18° and compared with each other and then the next two days with newly prepared electrodes of the same composition. All the examined electrodes agreed within 0·0001 volt.

When we tried to use the simple quinhydrone electrode in a phosphate mixture of p_H 6·79 containing glucose, we observed that the potentials between electrodes with and without glucose were not steady but decreased gradually. In Fig. 6 are shown curves representing our measurements with solutions containing 10 g. glucose (curves I and II), 5 g. (curve III) and 1 g. (curve IV) in 100 cc. The concentration of quinhydrone was 0·0025 *M*.

The shape of the curves shows that we have not to do with a reaction between glucose and quinhydrone, the change in π having a higher velocity in the more dilute glucose solutions than in the more concentrated ones. By and by we convinced ourselves that the phenomenon is a specific influence of glucose on the platinum. In order to test this explanation we made the following two experiments.

1. Two platinum electrodes were kept for 3 hours at 18° in a solution of 10 g. glucose in 100 cc. and used for the measurement of phosphate p_H 6·79 + 10 g. glucose in 100 cc. + quinhydrone against the same mixture without glucose. The measurements are shown in Fig. 7, curve I.

2. Two platinum electrodes were kept for 1 hour at 18° in a phosphate mixture p_H 6·79 containing quinhydrone (0·0025 *M*) and then used for the measurement of the mixture with glucose (10 g.) against the mixture without glucose. Curve II, Fig. 7, represents the measurements.

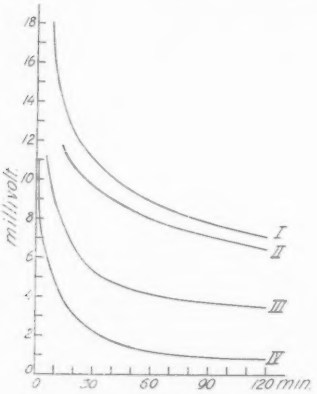

Fig. 6.

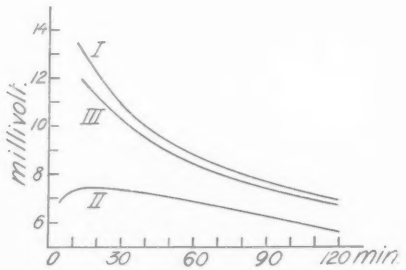

Fig. 7.

In all the electrodes the quinhydrone was 0·0025 *M*.

The single potentials of two electrodes, in each individual case corresponding to the curves I and II, agreed well; the maximum discrepancies of the observations from the corresponding curve values were 0·8 millivolt, and most of the observations agreed within 0·2–0·4 millivolt with the curve values. But in shape the two curves are not at all like each other as will be seen from Fig. 7, in which is also drawn a curve III representing the average of the curves I and II in Fig. 6. The agreement of curves III and I in Fig. 7 is very remarkable. Evidently, in these cases, the fall of π must have the same origin. A similar influence of glucose was observed neither in the numerous measurements we have made with the hydroquinhydrone electrode and phosphate or bicarbonate solutions, nor in the solutions of hydrochloric acid. We point out that Warburg [1922, p. 247] observed that hydrogen electrodes in bicarbonate solutions containing saccharose did not establish the potential as rapidly as in the pure salt solutions. We shall not further discuss this rather surprising phenomenon.

Summary.

1. The influence of glucose and of alcohol on the p_H of a phosphate mixture, p_H 6·79, is examined by means of the hydroquinhydrone electrode. Glucose makes the mixture more acid, and alcohol makes it less acid. The influences of glucose and alcohol are additive.

2. Solutions of secondary sodium phosphate and sodium bicarbonate are examined with the hydroquinhydrone electrode after saturation with carbon

dioxide at barometric and higher pressures. The influence of glucose on the p_H values of the liquids saturated with carbon dioxide was examined and found to be very feeble.

3. A hydroquinhydrone electrode in a phosphate mixture, p_H 6·79, was found to be very stable and suitable as a comparison electrode for measurements with hydroquinhydrone electrodes.

4. A hydroquinhydrone electrode in 0·01 *N* HCl, 0·09 *N* KCl is not very stable. It was found that it was stabilised by addition of glucose.

5. It was found that the simple quinhydrone electrode cannot be applied to phosphate solutions containing glucose. A specific action of the glucose on the platinum of the electrode was observed.

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